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### ► To cite this version:

Mélanie Fouesnard, Johanna Zoppi, Mélanie Pétéra, Léa Le Gleau, Carole Migné, et al.. Dietary switch to Western diet induces hypothalamic adaptation associated with gut microbiota dysbiosis in rats. *International Journal of Obesity*, 2021, 45 (6), pp.1271-1283. 10.1038/s41366-021-00796-4 . hal-03171983

**HAL Id: hal-03171983**

**<https://hal.science/hal-03171983>**

Submitted on 7 Sep 2021

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

Running title: Western diet and rat microbiota-hypothalamus axis

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Authors have no financial or personal conflict of interest to declare.

## Abstract

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

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

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

**Conclusion:** A potentially pro-inflammatory gut microbiota and an early hypothalamic oxidative stress appear shortly after WD introduction. Tripartite data integration highlighted putative links between gut microbiota sub-networks and hypothalamic oxidative stress. Together with the absence of hypothalamic modifications in germ-free rats, this strongly suggests the involvement of the microbiota-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 <sup>1</sup>, mainly regulated by the coordinated action of many brain regions including the brainstem,  
52 the hypothalamus and cortical or subcortical brain areas <sup>1</sup>. Among them, the hypothalamus integrates  
53 internal peripheral signals mostly from the gastro-intestinal tract as well as from adipose tissue or  
54 pancreas <sup>2</sup>. It also integrates brainstem information originating from the vagus nerve and circulating  
55 peripheral signals to adapt energy intake and/or energy expenditure <sup>3-6</sup>.

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 <sup>7</sup>. Indeed, although continuous  
60 exposure to WD leads to either no effect on energy intake <sup>8,9</sup>, hyperphagia in some studies <sup>10,11</sup> or  
61 sporadic spikes of hyperphagia in others <sup>12</sup>, 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 <sup>13</sup>, followed  
63 by a progressive normalization of energy intake within a week <sup>7,14,15</sup>. 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 <sup>7,16</sup>.  
66 Moreover, 1 to 3 days of WD consumption induces hypothalamic inflammation, as demonstrated by  
67 increased mRNA levels of pro-inflammatory cytokines <sup>17,18</sup>. In mice, this early phase is immediately  
68 followed by reactive gliosis, *i.e.* astroglial and microglial activation <sup>19-21</sup>, 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 <sup>17,21</sup>. Interestingly, diet-induced obesity (DIO)-resistant mice  
72 display early hypothalamic inflammation and gliosis but with a lesser intensity <sup>19</sup> and a different pattern

73 compared to DIO-prone mice<sup>22</sup>. 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 consumption<sup>23</sup>, 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<sup>24,25</sup>.

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<sup>26,27</sup>. 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 stress<sup>28</sup>. 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 death<sup>29</sup>. 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<sup>30,31</sup>. 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  
91<sup>32</sup>, 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 microorganisms<sup>33</sup>. Long-term WD consumption induces a  
95 decrease in gut microbiota diversity and profound compositional changes<sup>8,34</sup>. 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

Human microbiota-colonized mice<sup>35</sup>. Thus, we hypothesized that acute dietary switch from chow to WD results in early changes in microbiota composition and/or function that participates directly or indirectly to early hypothalamic inflammation in rats. To test this hypothesis, we determined how hypothalamic response as well as the gut microbiota composition and activity evolved during the first days following WD exposure, using 16S rRNA gene sequencing and caecal content and hypothalamic metabolomics analysis in conventional rats. Data integration tools were then used to highlight the potential links between microbiota changes and hypothalamic metabolic pathways associated to a pro-inflammatory context. Lastly, we used germ-free rats to investigate the potential connection between the gut microbiota and the early adaptation of hypothalamus to WD.

## Methods

### Animals

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

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

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

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

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

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

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

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

#### **Determination of caecal microbiota composition**

Total DNA extraction and V3-V4 PCR were performed as already described <sup>36</sup>.

#### **Hypothalamus and caecal content metabolomic profiles**

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

#### **Hypothalamic gene expression levels**

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

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



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

## Statistics and data integration

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

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

## Data availability

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

## Results

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

Several studies have shown that the intensity of the hyperphagic phase at normal weight following WD introduction is predictive of propensity to develop obesity on the long term<sup>24,25</sup>. We confirmed that rats switched to WD displayed a first phase of increased energy intake followed by normalization of energy intake (Fig. 1 A (kcal.g<sup>-1</sup> of body weight) and Supplementary Fig. 1 (kcal)). After 6 weeks of WD, they exhibited greater body weight gain (Fig. 1B), final body weight (572±15 vs. 513±11g, 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 µg.ml<sup>-1</sup>, P=0.02) and a tendency for greater fasted glycemia (275.0±46.7 vs. 166.6±18.1 mg.dl<sup>-1</sup>, P=0.06). We also confirmed that first week energy intake was positively correlated to final body weight (Fig. 1C) and visceral adiposity (Fig. 1D). Interestingly, first week energy intake was also positively correlated to fasted glycemia (Fig. 1E) and plasma LBP (Fig. 1F) measured at week 6. Thus, the intensity of this phase of increased energy intake upon dietary switch to WD seems predictive of later obesity parameters but also of metabolic and systemic inflammatory parameters.

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

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

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

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

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

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

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

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

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

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

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

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

## Discussion

### Oxidative stress: an early hypothalamic response to WD exposure

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

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

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

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

Caecal microbiota composition and activity were highly and rapidly impacted by the dietary switch. Caecal microbiota composition evolved towards a pro-inflammatory bacterial environment with an increase in *Desulfovibrionaceae* (Proteobacteria) relative abundance within 24h. Proteobacteria as Gram-negative bacteria and *Desulfovibrionaceae* as sulfate-reducing bacteria contribute to inflammation in the intestinal epithelium <sup>34,51</sup>. *Tannerellaceae* abundance increased in a similar manner. *Tannerellaceae* are endotoxin-producers and a member of this family, *Parabacteroides distasonis*, has been reported to be a microbial marker of inflammatory bowel disease in human <sup>52</sup>. Two days of WD consumption induced a drastic decrease of *Lactobacillaceae* relative abundance. *Lactobacillaceae*, and particularly members of the *Lactobacillus* genus, participate to the maintenance of intestinal barrier integrity by modulating tight-junction proteins expression <sup>53</sup>. Their decrease could contribute to bacteria and/or pro-inflammatory bacterial components passage across the intestinal epithelium, leading to systemic inflammation.

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

Unlike in conventional rats, dietary switch from chow to WD in GF rats did not impact expression levels of genes involved in oxidative stress and inflammation. In conventional rats, the hypothalamic inflammatory response to WD remained modest since only IL-6 expression was upregulated. Hypothalamic inflammatory response to WD has also been associated by others to a modest increase in expression of IL-6 after 1d to 3d of WD and no change in IL-1 $\beta$  and TNF- $\alpha$ <sup>17,54,55</sup>. The lack of change in the GF rats suggests that the presence of gut microbiota participates to the early hypothalamic stress encountered shortly after WD introduction.

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



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

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

#### **List of abbreviations**

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

#### **Acknowledgments**

We thank Mathilde Guerville and Annaëlle Sinquin as well as Helen Raybould. We thank Isabelle Nogret for her technical help. Caecal Metabolomic analysis were performed within the metaboHUB French infrastructure (ANR-INBS-0010). This work was partly funded by a grant from INRAE AlimH division.

### Competing Interests

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

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

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## Figures legends

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

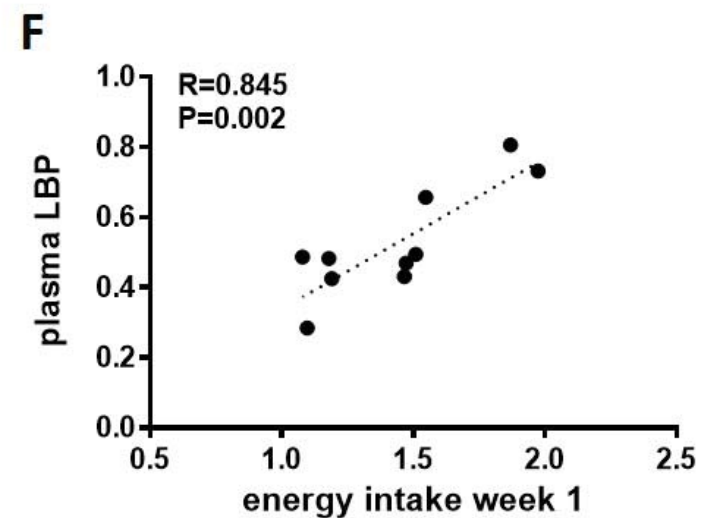
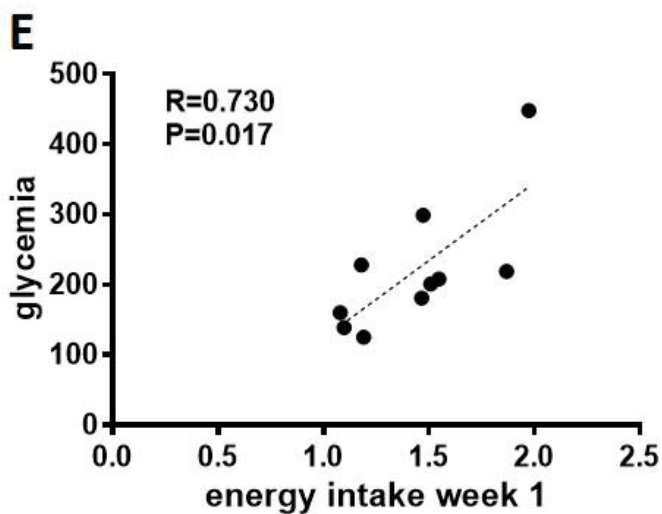
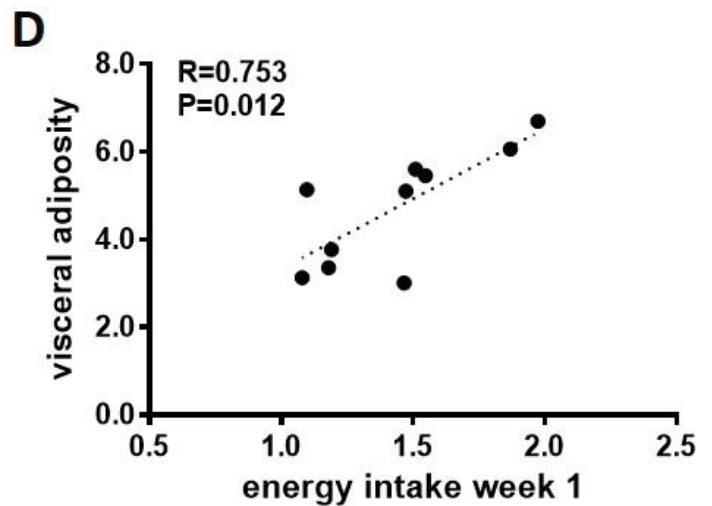
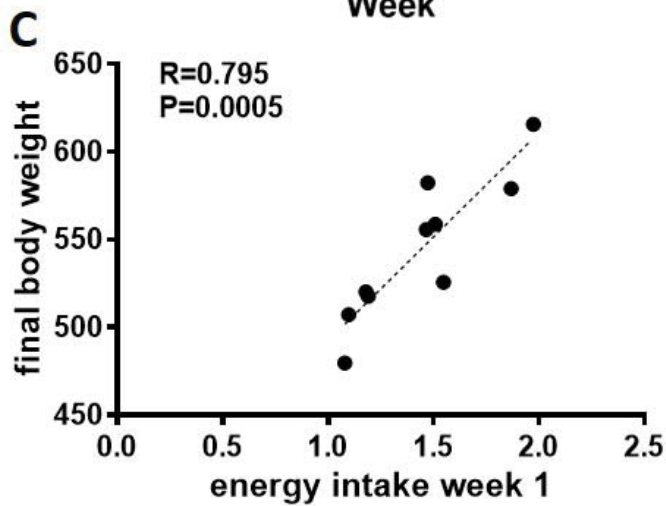
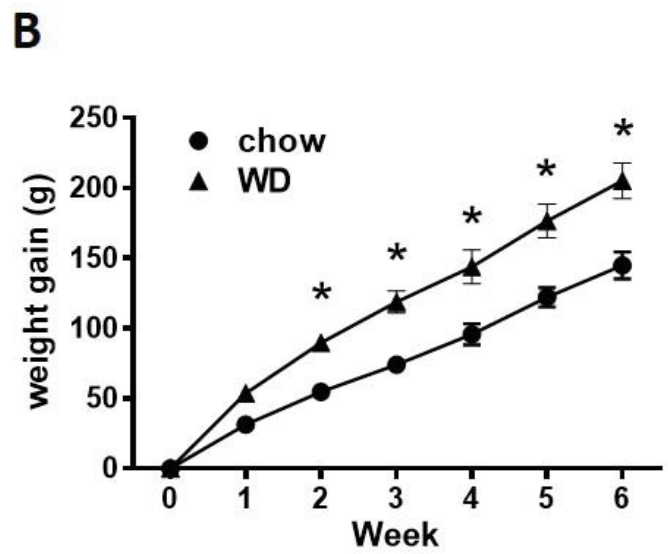
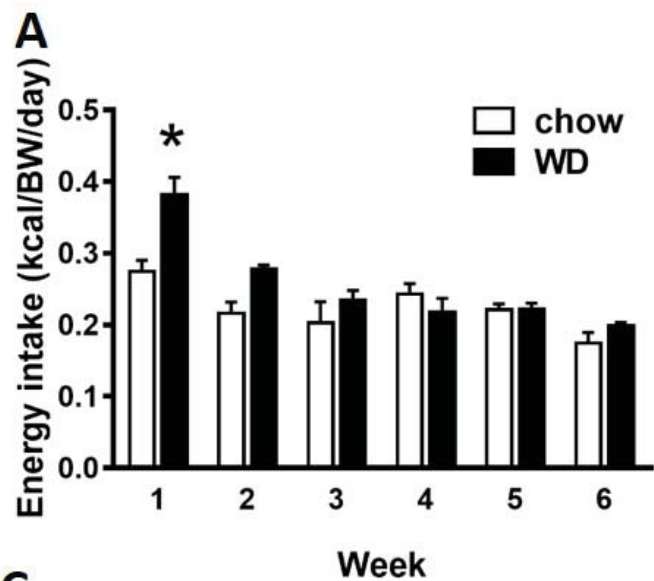
**Figure 2. Hypothalamic metabolome evolution during adaptation to WD in conventional rats.** Evolution of hypothalamic metabolome represented by PLS-DA (first 2 components) after WD switch (A). Ellipses represent 95% confidence interval. N=6-7/group. Different letters show significant differences (PERMANOVA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

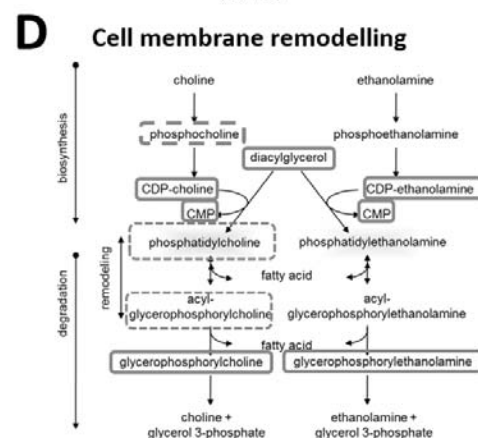
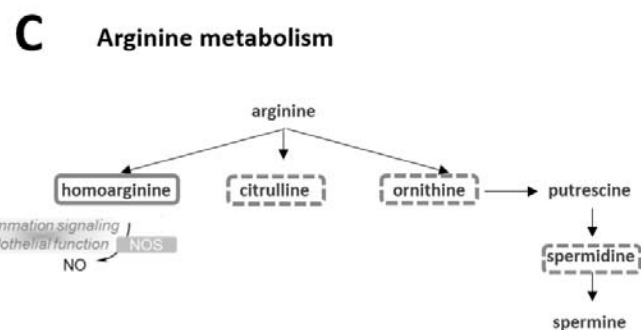
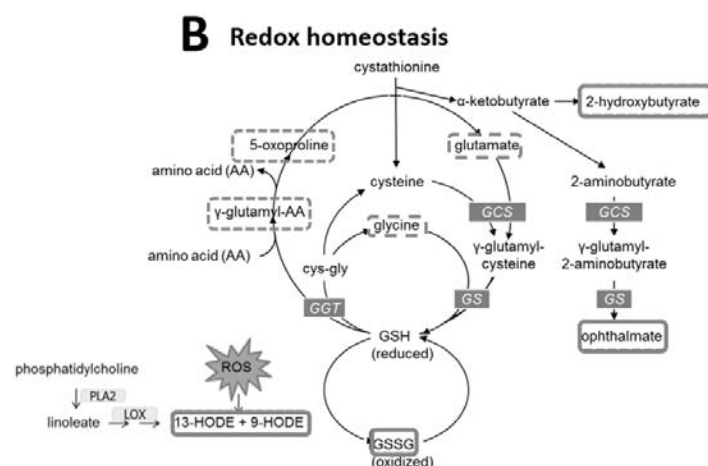
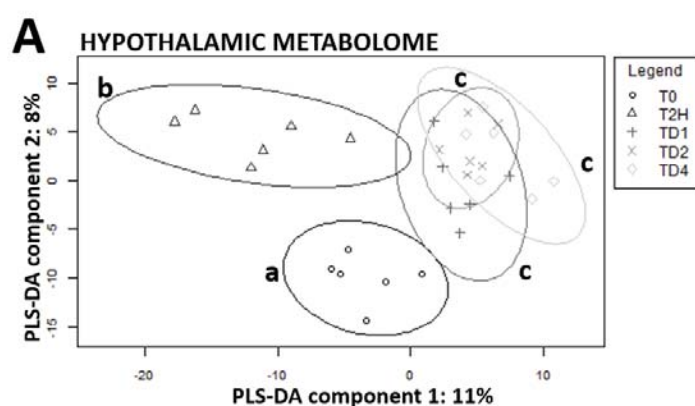
**Figure 3. Evolution of caecal microbiota diversity and composition during early adaptation to WD in conventional rats.** Evolution of  $\beta$ -diversity (PCoA on Bray-Curtis distance) and  $\alpha$ -diversity (Shannon index) (A), of *Desulfovibionaceae*, *Lactobacillaceae* and *Tannerellaceae* relative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

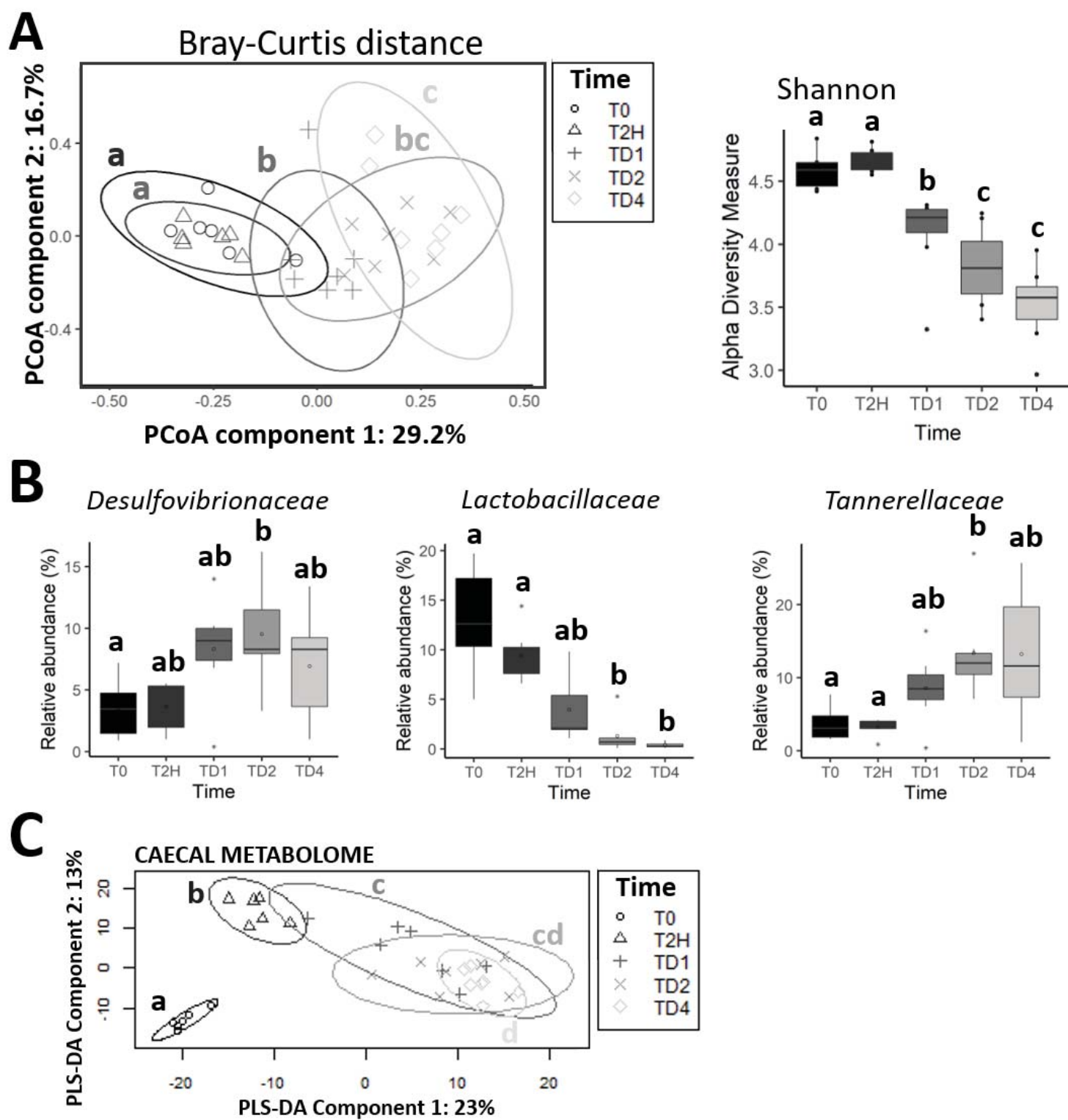
**Figure 4. Tripartite network showing links between caecal microbiota, caecal metabolome and hypothalamic oxidative stress.** Pathway enrichment showing how redox homeostasis is impacted by dietary switch is represented in the upper part of the figure. Correlations between hypothalamic metabolites,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

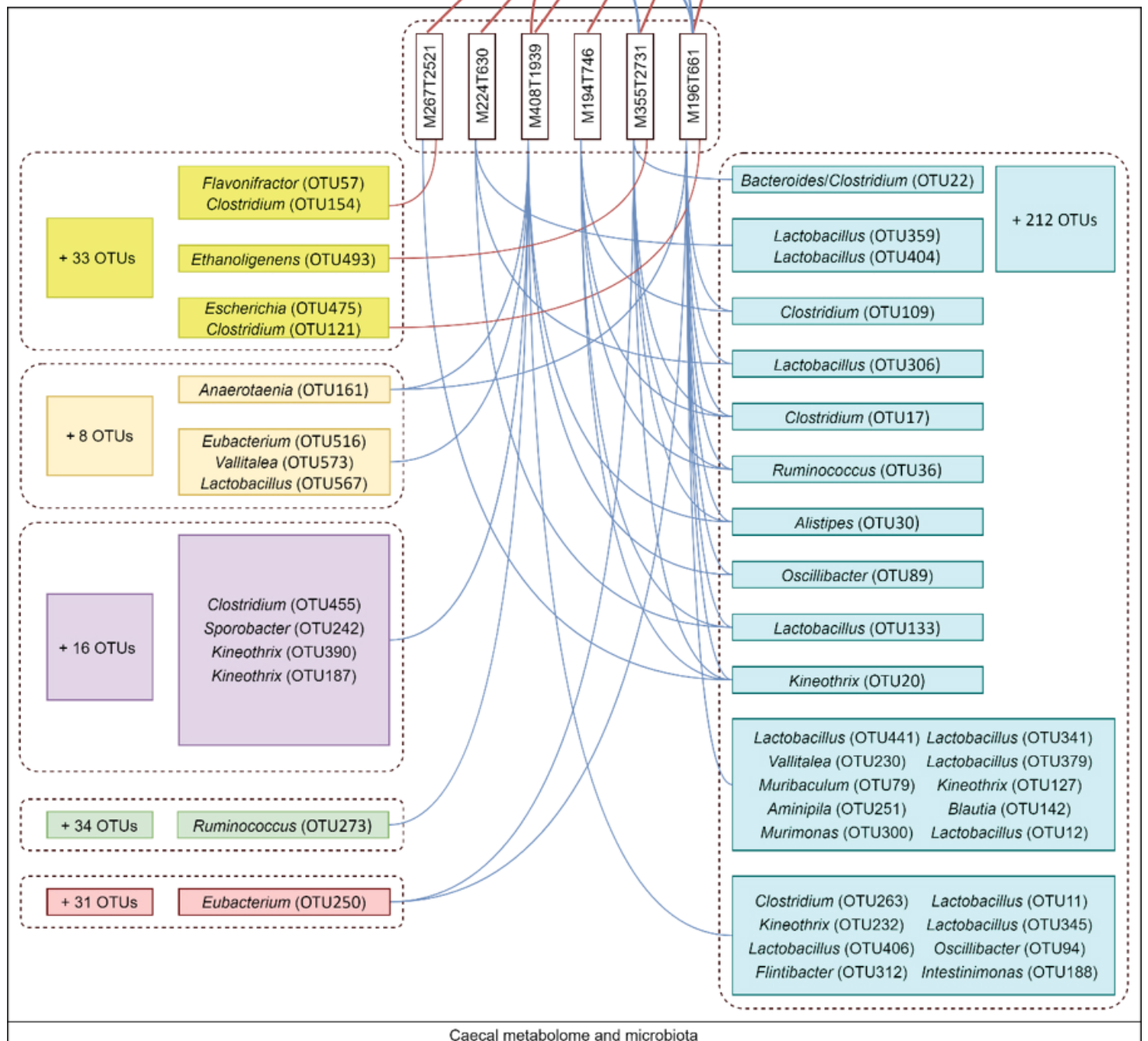
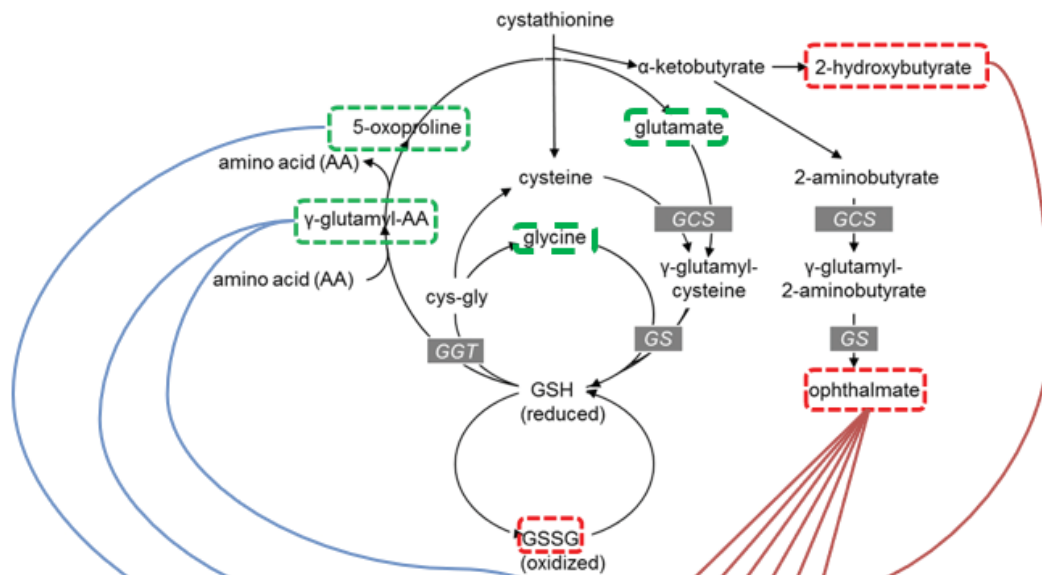
**Figure 5. Germ-free and conventional rat response to 2 days of WD consumption.** Rats daily energy intake (kcal.g of body weight/day) (A). Hypothalamic expression levels of genes involved in redox homeostasis maintenance (B). Hypothalamic expression levels of genes involved in inflammation (C). Data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



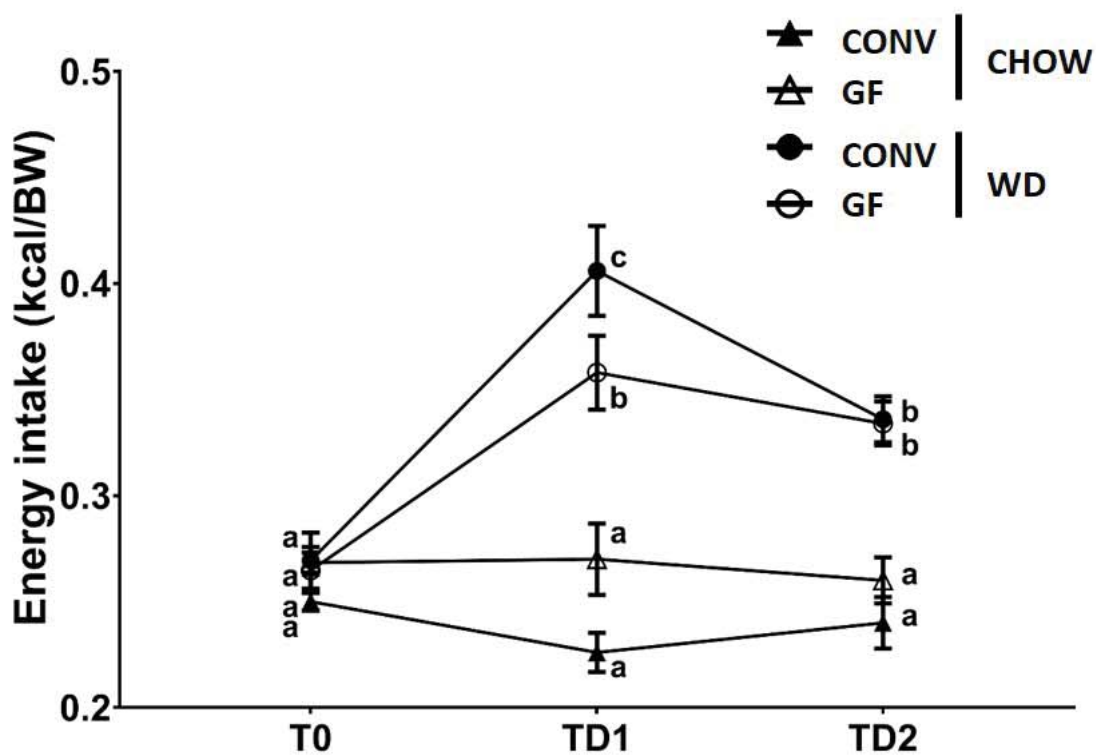
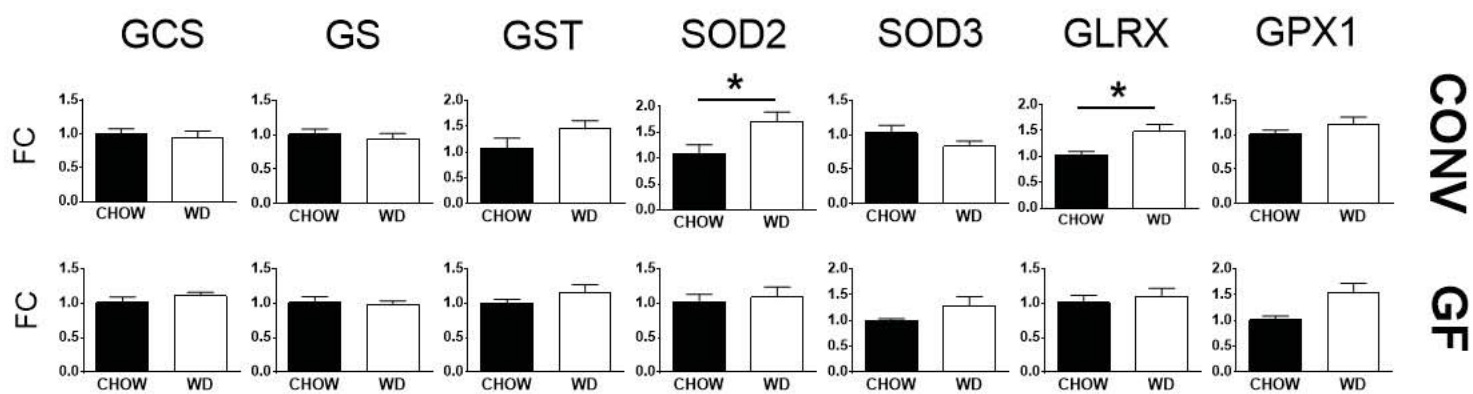








Caecal metabolome and microbiota

**A****B****C**