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Accepted manuscript

1 **Dietary Alpha-Lactalbumin Alters Energy Balance, Gut Microbiota Composition and**
2 **Intestinal Nutrient Transporter Expression in High-Fat Diet Fed Mice**

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35 **Abstract**

36 Recently there has been a considerable rise in the frequency of metabolic diseases, such as obesity,
37 due to changes in lifestyle and resultant imbalances between energy intake and expenditure. Whey
38 proteins are considered as potentially important components of a dietary solution to the obesity
39 problem. However, the roles of individual whey proteins in energy balance remain poorly
40 understood. This study investigated the effects of a high fat diet (HFD) containing alpha-
41 lactalbumin (LAB), a specific whey protein, or the non-whey protein casein (CAS), on energy
42 balance, nutrient transporters expression, and enteric microbial populations. C57BL/6J mice (n = 8)
43 were given a HFD containing either 20% CAS or LAB as protein sources or a low-fat diet (LFD)
44 containing CAS for 10 weeks. HFD-LAB fed mice showed a significant increase in cumulative
45 energy intake (P=0.043), without differences in body weight, energy expenditure, locomotor
46 activity, respiratory exchange ratio or subcutaneous and epididymal adipose tissue weight. HFD-
47 LAB intake led to a decrease in the expression of glucose transporter *glut2* in the ileum (P=0.05)
48 and in the fatty acid transporter *cd36* (P<0.001) in both ileum and jejunum. This suggests a
49 reduction of absorption efficiency within the small intestine in the HFD-LAB group. DNA from
50 faecal samples was used for 16S rRNA-based assessment of intestinal microbiota populations; the
51 genera *Lactobacillus*, *Parabacteroides* and *Bifidobacterium* were present in significantly higher
52 proportions in the HFD-LAB group. These data indicate a possible functional relationship between
53 gut microbiota, intestinal nutrient transporters and energy balance, with no impact on weight gain.

54 **Introduction**

55 Obesity has emerged as one of the most prevalent global health problems over the last 30 years
56 because of its association with comorbidities such as chronic inflammations, type II diabetes,
57 cardiovascular disorders and certain types of cancer[(1)]. Whilst the aetiology of obesity is
58 multifactorial, there is a growing recognition that high energy density diets (containing high levels
59 of fat and sugar) are major contributors to long-term imbalance between energy intake and energy
60 expenditure[(2)], with comparable effects seen in rodents including mice such as the C57BL/6J strain.
61 Indeed, energy dense diets increase intestinal energy absorption and fat storage in adipose tissue[(3)].
62 Additionally, these diets impair the hypothalamic regulation of hormones involved in the control of
63 energy balance[(4)]. Thus, one approach to reducing weight gain or causing weight loss would be to
64 devise interventions that affect the cross-talk between the gut, adipose tissue and the hypothalamic
65 mechanisms regulating energy balance.

66 Nutrient digestion and absorption provides the necessary energy for the survival of living organisms
67 and the gastrointestinal (GI) tract has evolved to optimise these processes[(5)]. In particular, nutrient
68 absorption takes place in the duodenum, jejunum and ileum, through paracellular movements or by

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69 uptake through specific nutrient transporters, which are able to transport fats (e.g., Fatty Acid
70 Transporter, FATP4; Cluster Differentiation 36, CD36), sugars (i.e. Glucose Transporter 2,
71 GLUT2; Sodium-Glucose transporter Protein 1, SGLT1) and amino acids (i.e. L-type Amino acid
72 Transporter 4, LAT4; neural amino acid transporter 1, B^0AT1) into the blood stream[6].
73 The complexity of nutrient absorption is further highlighted by the network of diverse intrinsic and
74 extrinsic factors regulating this process. With regard to intrinsic factors, it is notable that deficiency
75 of the anoretic hormone leptin in mice[6] increases energy ingestion leading to a greater adiposity
76 and weight gain, coupled with associated hypothalamic-neuropeptide changes and chronic intestinal
77 inflammation[7]. Similarly, targeted deletion of the gene encoding GLUT2, a key intestinal glucose
78 transporter, also increases energy intake[8].
79 Of the extrinsic factors, the gut microbiota plays a key role in harvesting energy from ingested food
80 and providing it for host metabolism[9; 10; 11; 12]. Hence, germ free mice are protected against obesity
81 despite consuming more calories than control mice, but this phenotype is reverted after faecal
82 microbiota transplantation from conventionally raised mice[13]. Using gnotobiotic mice fed a high-
83 fat or low-fat diet (HFD and LFD, respectively), a role has been suggested for *Clostridium*
84 *ramosum* in the upregulation of body fat deposition promoting factors[14]. These data, coupled with
85 the observed impact of gut microbiota on brain formation, including hypothalamic micro-
86 structures[15], provide novel avenues for modulating the activity of the intestine, adipose tissue and
87 hypothalamus, involving microorganisms.
88 Alpha-lactalbumin (LAB) is a globular protein well known as a source of peptides having beneficial
89 properties such as antioxidant bioactivity and immune modulation ability[16]. It constitutes around
90 25% of the bovine milk whey proteins[16]. Whey can be extracted during the manufacture of cheese
91 and comprises also other protein types, varying in abundances, including β-lactoglobulin (~ 65%),
92 bovine serum albumin (BSA, ~8%), lactoperoxidase (0.25-0.5%), lactoferrin (Lf, ~1%) as well as
93 other minor proteins such as immunoglobulins (<1%)[17]. Recently, whey proteins have been
94 considered as a potential dietary solution to obesity in light of the discovery that these proteins, as
95 an isolate (WPI) or concentrate (WPC), acutely increases the production of hormones that are
96 involved in satiety in both humans and rodents[18; 19]. Moreover, intake of whey proteins in humans
97 reduces fat absorption[20]. This effect might be attributed to an altered composition of the gut
98 microbiota involved in energy harvest, since other studies have shown that dietary whey proteins
99 can influence the composition of the gut microbiota[21; 22; 23; 24]. Indeed, we showed that WPI
100 reduced the proportion of Firmicutes in the mouse gut microbiome[22], and this was accompanied by
101 a reduction in the expression of intestinal nutrient transporters, specifically *glut2* and *fatp4*. . In
102 addition, we showed that WPI reduced epididymal adipose tissue (eWAT) weight and overall body

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103 weight gain. Consistent with changes in the gut, WPI increased energy intake, which was reflected
104 in the altered hypothalamic gene expression of neuropeptides, namely proopiomelanocortin (*pomc*),
105 neuropeptide Y (*npy*) and ghrelin[22].
106 While these data suggest a modulatory effect of WPI on the gut microbiome, hypothalamus and
107 adipose tissue, it was not clear if the changes in energy intake were driven directly from the
108 gut/hypothalamus axis or were secondary to the changes in adiposity. Indeed, previously it was
109 shown that specific whey proteins, such as BSA and Lf, influenced in a different way body weight,
110 energy intake and plasma leptin level[25; 26].
111 Given that LAB has been shown to affect the energy balance but the underlying mechanism is still
112 unclear[27; 28], in the current study we sought to further investigate the link established previously
113 between gut-hypothalamic-adipose control of energy balance using LAB as the main protein source.
114 The protein was given in a physiological amount (i.e. 20% calories) during HFD-induced weight
115 gain phase in the C57BL/6J strain of mice, which was used as the model for humans. LAB was
116 introduced early in development (5 weeks of age), given WPI specificity to influence weight gain
117 during this period[29]. As the control, we fed mice a LFD or HFD containing the same physiological
118 amount of a non-whey milk protein, casein.
119
120
121
122 **Material and Methods**
123 **Experimental strategy**
124 The *in vivo* experiments were approved by the University College Cork Animal Experimentation
125 Ethics Committee (2011/005) and were licenced under the European Directive 2010/63/EU.
126 Twenty-four C57BL/6J three week old male specific pathogen free mice were purchased
127 commercially (Harlan; UK) and were singly housed in each cage with enrichment (Litaspen 8/20
128 and alpha-dry Plus bedding; LBS-biotech, UK) on a 12 h light/dark cycle with humidity maintained
129 at 45-60% and temperature between 19-22°C. The mice had *ad libitum* access to food and water
130 throughout the study unless otherwise stated. The health of the animals and the environment
131 parameters were checked and recorded daily. No adverse effects were observed by the dietary
132 interventions or procedures detailed below. The mice were provided with a diet containing 10%
133 (low) fat and 20% casein (LFD-CAS; #D12450Bi; Research Diets, USA) (all % values by energy)
134 during the initial 2 week acclimatisation period, and then weight matched mice were switched to a
135 45% (high) fat diet containing either 20% casein (HFD-CAS; #D12451i) or alpha-lactalbumin
136 (HFD-LAB; #D13081701i) (n=8 per group). The controls continued to receive LFD-CAS (n=8).

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137 The diets were OpenSource and were made by Research Diets, USA, with LAB sourced from
138 Sigma, USA (diets composition details, Table S1). Body weight as well as food intake were
139 measured weekly. The latter was converted to gross energy intake using the dietary energy density
140 of the diets (HFD=19.79 KJ/g and LFD=16.11 KJ/g; Research Diets, USA).
141 During weeks 8 and 9, mice were placed in TSE Phenomaster cages (Germany) for 3 days to
142 measure metabolic parameters, specifically, oxygen consumption (ml/h, VO2), carbon dioxide
143 production (ml/h, VCO2), respiratory exchange ratio (VCO2/VO2, RER) and locomotor activity
144 (X, Y and Z planes). The data were collected in the final 24h of the housing period as detailed
145 previously[30].
146 At week 10, mice were fasted for 10-12h commencing at 22.00 in the dark phase, then
147 anaesthetized (100mg/Kg Ketamine and 10 mg/Kg Xylazine) and blood samples were collected.
148 Mice were sacrificed by cervical dislocation and tissues were collected and samples were stored at -
149 80˚C for subsequent analysis. The length of the small intestine and the weight of all the tissues were
150 recorded on fresh tissues before snap freezing them.
151

152 **DNA Sequencing**

153 Faecal samples were collected directly from the colon, homogenized and processed using
154 mechanical and chemical lysis. The 16S rRNA gene (V3-V4 region) was amplified with Universal
155 primers (PCR1 Forward and Reverse primer as to the Illumina 16S Metagenomic Sequencing
156 Protocol) that facilitated sequencing on the Illumina MiSeq platform (2x250bp paired-end reads; V3
157 sequencing chemistry). The fastq files were filtered on the basis of quality (removal of low quality
158 nucleotides at the 3' end) and length (removal of sequences with less than 200nt) with prinseq[31],
159 and paired reads with a minimum overlap of 20 bp were joined using Fastq-join[32].

160 Sequences were clustered (97% identity) to obtain Operative Taxonomic Units (OTUs) using
161 closed-reference usearch v7.0 algorithm[33] and chimeric OTUs were removed through use of the
162 gold database. The taxonomic assignment of OTUs was obtained using the Ribosomal database
163 project (RDP)[34]. Alpha and beta-diversity was determined using QIIME[35], and additional
164 analyses were performed with the R package phyloseq[36].

165 To identify chow-associated microbes, total DNA was extracted from ~ 10 g of chow containing
166 20% fat and 20% casein (LFD-CAS; #D17052702; Research Diets, USA), and ~ 10 g of chow
167 containing 40% fat and 20% casein (HFD-CAS; #D17052705; Research Diets, USA) using the
168 Gene All Extragene SoilSV kit (GeneAll). The Qubit high-sensitivity DNA assay (Bio-Sciences,
169 Dublin, Ireland) was used for accurate quantification of the total DNA. Whole-metagenome shotgun
170 libraries preparation was performed following the Nextera XT DNA library preparation guide from

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171 Illumina[37] and sequencing was performed using the Illumina NextSeq 500 with a v2 NextSeq
172 500/550 high-output reagent kit (300 cycles).

173 The raw shotgun metagenomic sequences were filtered on the basis of quality (removal of low
174 quality nucleotides at the 3' end and application of sliding window trimming, cutting once the
175 average quality within the window falls below a treshold quality of 20 bp) and length (removal of
176 sequences of less than 200nt) with prinseq[31]. The filtered sequences were then converted to bam
177 files using SAMtools[38], and duplicate reads were subsequently removed using Picard Tools
178 (https://github.com/broadinstitute/picard). The quality of the sequences was tested using SAMtools
179 in combination with Picard Tools, removing the low quality sequences. Taxonimic analysis was
180 performed using Kaiju[39].

181 **Gene expression**

182 The small intestine samples, stored at -80˚C, were initially immersed in a RNAlater-Ice frozen
183 tissue transition solution and stored at -20˚C according to the instructions of the manufacturer
184 (Ambion). This step allows untangling of the intestine while preserving the integrity of the RNA so
185 that tissue samples, corresponding to the ileum (1 cm from the distal end of the small intestine) and
186 jejunum (the central part of the small intestine), could be cut.
187 Total RNA was extracted from the intestinal samples and hypothalamic blocks using RNeasy
188 Minikit and QIAshredder columns (Qiagen), and from epididymal adipose tissue (eWAT) and
189 subcutaneous adipose tissue (sWAT) using QIAzol Lysis Reagent (Qiagen). The extracted RNAs
190 were treated with DNase (Qiagen, Ireland). Complementary DNA was synthetized from 600ng total
191 RNA using SuperscriptTM II Reverse Transcriptase kit (Life Technologies, Ireland), and subjected
192 to Real-Time PCR (Roche, Ireland) using SYBR Green Select Master Mix (Roche, UK) as detailed
193 before[40]. The gene expression was calculated using 2-ΔΔCp and normalised against the reference
194 gene β-actin (intestine and adipose tissue) and YWAZ (hypothalamus). The sequence of the primers
195 can be found elsewhere[25; 30; 40].
196

197 **Plasma leptin, glucose and triglyceride levels**

198 Plasma leptin and glucose levels were determined using Mouse Leptin ELISA kit (Crystal Chem,
199 USA) and Mouse Glucose Assay (Crystal Chem, USA) respectively. Triglyceride (TAG) level in
200 the plasma was measured using Triglyceride Quantification Assay Kit (abcam, UK).
201

202 **Statistical analysis**

203 For power calculation, the coefficient of variation was measured using data (body weight gain)

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204 from a previous study[40] and determined to be 8.8%. For a power of 80% and a significance level of
205 5%, this allows detection of a difference of 14% with a sample size of 8 mice per treatment. Body
206 weight and energy intake differences over 10 weeks were analysed by a two-way repeated-measures
207 ANOVA with Bonferroni's *post hoc* pairwise comparisons. Statistical analysis of gene expression,
208 cumulative energy intake, intestine weight/intestine length, plasma leptin and triacylglycerol data
209 were performed using one-way ANOVA followed by pairwise comparison using Bonferroni's *post*
210 *hoc* test. Non-parametric data were compared by Kruskal-Wallis ANOVA followed by Mann-
211 Whitney *U* test. Data were expressed as mean ± SEM and significance was set at P<0.05 using
212 SPSS software version 24 (IBM Corp). V_{O2} and heat production was analysed by ANCOVA (SAS
213 software version 9.3), with total body weight being used as the covariant.
214 Gut microbiota data were statistically analyzed by Adonis and Anosim for beta-diversity analysis.
215 ANOVA was used to calculate significance for alpha-diversity-related analyses (statistical
216 significance established at P<0.05). Statistical differences across multiple samples were determined
217 by Kruskal-Wallis and False discovery rate (FDR, qvalue) control based on the Benjamini-
218 Hochberg procedure, used to correct for multiple testing with the R statistical package
219 (https://www.r-project.org/). Test for association across all groups of samples were performed
220 through correlation analysis. This was based on Spearman's rank correlation test by the R function
221 of cor.test, which aimed to examine similar expression association profiles between groups of
222 samples (https://www.r-project.org/package=corrplot) and adjustment of the Pvalue by the
223 Benjamini-Hochberg method. Statistical significance was established at P<0.05 (*), with other
224 ranges, i.e. P<0.01 (**) and P<0.001 (***), also being noted.

225 **Results**

226 **LAB increased cumulative energy intake but not weight gain**

227 Mice fed HFD-LAB and HFD-CAS had similar body trajectories (Fig. 1A) but differed from the
228 LFD-CAS group in the time taken to increase weight. Notably, the HFD-CAS group had a
229 significantly higher weight than LFD-CAS by week 3 (P=0.033) while the weight of the HFD-LAB
230 group did not significantly exceed that of the LFD-CAS group until week 6 (P=0.045)
231 ($F_{(8,160)}$=440.2, P<0.001 for the effect of the Time, $F_{(2,20)}$=5.5, P<0.05 for the effect of the Diet,
232 $F_{(16,160)}$=11.6, P<0.001 for the effect of the Time x Diet interaction) (Fig. 1A). Despite having
233 similar body weight trajectories, the energy intake of HFD-LAB showed a trend towards an
234 increase at week 2 (P=0.053) (413±9 kJ in HFD-LAB, 383±8 kJ in HFD-CAS and 293± 8 kJ in
235 LFD-CAS) and it was higher in HFD-LAB compared to HFD-CAS at week 4 (P=0.037)
236 ($F_{(7,140)}$=7.2, P<0.001 for the effect of the Time, $F_{(2,20)}$=81.7 , P<0.001 for the effect of the Diet,
237 $F_{(14,140)}$=1.3 , P>0.05 for the effect of the Time x Diet interaction) (Fig. 1B). The cumulative energy

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238 intake of HFD-LAB was significantly increased by week 10 relative to HFD-CAS (P=0.043) (Fig.
239 1C). The difference in energy intake was reflected in the hypothalamic expression of neuropeptides.
240 Notably, at week 10, the expression of the anorexigenic neuropeptide *pomc* was reduced relative to
241 the LFD-CAS control (P=0.043 compared to HFD-CAS and P=0.001 compared to HFD-LAB),
242 without showing significant difference between the two HFD groups. (Fig. 1D). While the gene
243 expression for the orexigenic neuropeptides ghrelin and *npy* remained unchanged, the gene
244 expression of growth hormone secretagogue receptor (*ghsr*) was reduced in both HFD groups
245 compared to LFD-CAS (P=0.012 relative to HFD-CAS and P=0.001 relative to HFD-LAB) (Fig.
246 1D). Similar to *pomc* expression, the expression of *ghsr* between HFD-CAS and HFD-LAB groups
247 did not change. There was no effect due to diet on the expression of the fatty acid synthase (*fasn*).
248 The mismatch between energy intake and weight gain was unrelated to energy expenditure as both
249 HFD-CAS and HFD-LAB groups had similar 24h VO_2 levels (97 ±3 in HFD-LAB, 95±3 in HFD-
250 CAS and 101±3 in LFD-CAS; all values in ml/hr) and heat production (2.1±0.05 in HFD-LAB,
251 2.0±0.05 in HFD-CAS and 2.2±0.06 in LFD-CAS; all values in kJ/hr), where both parameters were
252 measured during week 8 and 9 period. There were no differences between groups in terms of 24h
253 locomotor activity (303±28 in HFD-LAB, 293±29 in HFD-CAS and 365±37 in LFD-CAS). While
254 the HFD-CAS and the HFD-LAB groups had similar RER, the corresponding values were lower
255 compared to LFD-CAS (both with P<0.001), consistent with increased fat consumption by former
256 groups (0.816±0.014 in HFD-LAB and 0.809±0.014 in HFD-CAS versus 0.916±0.008 LFD-CAS).
257

258 **LAB decreases the intestinal expression of glucose and fatty acid transporters**

259 To find an explanation for the apparent energy loss in the HFD-LAB group, the impact on gastro-
260 intestinal size and related gene expression of several nutrient transporters were measured. The
261 intestinal length was unaffected by the diet (33.2±0.61cm in HFD-LAB, 32.4±0.74 cm in HFD-
262 CAS and 32.9±0.38 cm in LFD-CAS). While the intestinal weight expressed relative to the length
263 and body weight was similar between HFD-CAS and HFD-LAB groups, this was significantly
264 lower compared to LFD-CAS controls (P=0.039 relative to HFD-CAS and P=0.019 relative to
265 HFD-LAB) (Fig. 2A). The data from ANCOVA analysis showed that there was not significant
266 effect of diet on intestinal weight/length when body weight was used as a covariant (0.024±0.001g
267 in HFD-LAB, 0.025±0.001g in HFD-CAS and 0.026±0.001g in LFD-CAS).
268 In the ileum, we observed a significant decrease in the expression of the sodium-glucose transporter
269 protein (*slgt1*) in both high fat groups compared to the LFD-CAS (P=0.026 relative to HFD-CAS
270 and P=0.029 relative to HFD-LAB), but *glut2* gene expression was specifically reduced in the
271 HFD-LAB compared to LFD-CAS (P=0.01) and it showed a trend towards a decrease, at the

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272 significance threshold, compared to HFD-CAS (P=0.053) (Fig. 2B). While *fatp4* gene expression
273 was unaffected in the ileum, the cluster of differentiation 36 (*cd36*; an integral membrane protein
274 responsible for importing fatty acids into the cell) was reduced in the HFD-LAB compared to the
275 LFD-CAS (P=0.001) and HFD-CAS (P<0.001) (Fig. 2B). The expression of genes encoding the L-
276 amino acid transporter, *lat4*, and the neutral amino acid transporter *slc6a19* was not significantly
277 affected (Fig. 2B).

278 The same gene expression was measured in the jejunum. Similar to the ileum data, *cd36* gene
279 expression in the jejunum was reduced in the HFD-LAB group compared to the other two groups
280 (P=0.001 relative to LFD-CAS and P<0.001 relative to HFD-CAS), whereas *glut2* expression did
281 not change across the groups (Fig. 2C). In addition, in the jejunum, the level of expression of *fatp4*
282 was significantly higher in the HFD-LAB group compared to the LFD-CAS control group
283 (P<0.001) and the expression of *lat4* showed a trend towards an increase in the HFD-LAB fed mice
284 relative to HFD-CAS fed mice (P=0.061) (Fig. 2C).

285 In light of the results obtained for *glut2* and *cd36* gene expression, we measured the levels of
286 glucose and triglyceride in the plasma. We observed that the glucose in the plasma of HFD-LAB
287 fed mice (311±23 mg/dL) was not significantly different to HFD-CAS (293±8 mg/dL) but was
288 lower compared to LFD-CAS fed mice (364±8 mg/dL, P=0.018). The level of triglyceride in the
289 HFD-LAB (37±4 mg/dL), HFD-CAS (35±6 mg/dL) and LFD-CAS (36±5 mg/dL) groups was not
290 significantly different.

291

292 **LAB does not affect leptin gene expression and plasma hormone availability**

293 Several tissues were harvested from the mice and their weights were recorded (Fig. 3A). The
294 weights of both the subcutaneous adipose tissue (sWAT) and the eWAT were greater in both the
295 HFD groups compared to the LFD-CAS control group (sWAT: P=0.002 relative to HFD-CAS and
296 P=0.056 relative to HFD-LAB; eWAT: P=0.001 relative to HFD-CAS and P<0.001 relative to
297 HFD-LAB) but there were no differences in the adipose tissues weights between HFD-CAS and
298 HFD-LAB (Fig. 3A). Next, the expression of several genes involved in the catabolism and
299 anabolism of fatty acids in the eWAT and sWAT was measured. In the current study, the expression
300 in the eWAT of genes involved in fatty acid catabolism (carnitine palmoitoyltransferase I, *cpt1*;
301 uncoupling protein, *ucp*; hormone-sensitive lipase, *hsl*; beta-3 adrenergic receptor *β-3ar*) and
302 anabolism (acetylCoA carboxylase 1, *acc1*; fatty acid synthase, *fasn*; lipoprotein lipase, *lpl*)
303 between HFD-LAB and HFD-CAS fed animals did not differ (Fig. 3B-C). There was also no
304 change in the expression of genes involved in fatty acids transport, such as *cd36*, *fatp1* and *glut4*
305 (Fig. 3C). However, consistent with the increased adiposity of the high fat fed groups, *acc1* gene

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306 expression was down regulated in HFD-CAS (P=0.008) and HFD-LAB (P=0.002) compared to
307 LFD-CAS (Fig. 3C). In addition, the inflammatory marker, cluster of differentiation 68 (*cd68*)
308 expression showed a trend towards an increase in the HFD-CAS group compared to the LFD-CAS
309 (P=0.057), but for the HFD-LAB group, the increase was not significant relative to LFD-CAS (Fig.
310 3C). The unchanged adipose tissues weights, coupled with gene expression data in the eWAT,
311 suggests an alternative mechanism of energy loss in the HFD-LAB group. We also measured leptin
312 (*ob*) gene expression in the eWAT to assess if there is a specific effect of LAB in leptin regulation,
313 similar to that attributed to the whey protein Lf[(26)].
314 Consistent with the gain in body weight and adipose tissue weight, the expression of the *ob* gene in
315 the HFD-CAS group increased compared to the control LFD-CAS (P<0.001) and, in the HFD-LAB
316 group, it showed a trend towards an increase relative to the LFD control (P=0.059), without
317 showing any differences between the two HFD groups (P=0.08) (Fig. 3C).
318 Similarly, no differences in *ob* expression were detected in the sWAT between the HFD groups
319 (Fig. 3E), and the expression of *fasn* and *glut4* genes were decreased compared with the LFD-CAS
320 group (*fasn*: P=0.001 relative to HFD-CAS and P=0.001 relative to HFD-LAB; *glut4*: P=0.021
321 relative to HFD-CAS and P=0.035 relative to HFD-LAB), with no difference between HFD-CAS
322 and HFD-LAB groups (Fig. 3E).
323 The *ob* gene expression in eWAT and sWAT was reflected in the plasma availability of leptin, as
324 the presence of LAB in the HFD did not influenced the plasma hormone level, relative to HFD-
325 CAS (Fig. 3D).
326
327 **Beta-diversity and gut bacterial composition differs due to LAB intake.**
328 After quality filtration and length trimming an average of 182,494 high-quality 16S rRNA
329 sequences per sample (i.e., faecal pellets collected from the colon) were obtained across two groups,
330 i.e., HFD-CAS (n=8) and HFD-LAB (n=8). Analysis of this data revealed that, while alpha-
331 diversity did not differ significantly across groups, a significant difference in beta-diversity
332 (P=0.002) between the microbiota of the HFD-CAS and HFD-LAB animals was apparent, as
333 represented by distinct clustering upon Principal Coordinates Analysis (PCoA) of all 16S rRNA
334 reads (clustered at 97% similarity) (Fig. 4A).
335 Differences in beta-diversity were reflected by taxonomic differences between the HFD-CAS and
336 HFD-LAB groups. At the phylum level, a lower Firmicutes/Bacteroidetes (ratio F/B) was apparent
337 in the HFD-CAS samples (ratio F/B=1.23) relative to the HFD-LAB equivalents (ratio F/B=1.42),
338 though significance was not achieved (Fig. 4B). At the family level, two families differed

339 significantly in a diet-associated manner. These were *Lactobacillaceae* (higher levels in HFD-LAB
340 samples) and *Streptococcaceae* (higher proportions in HFD-CAS samples) (Table 1).
341 At the genus level, statistically significant differences were apparent in the abundance of
342 *Parabacteroides*, *Bifidobacterium*, *Parvibacter* and *Lactobacillus*, all of which were present in
343 higher proportions in the HFD-LAB sample groups, as well as *Lactococcus*, *Roseburia* (P=0.021),
344 *Phascolarctobacterium* (P=0.018) and *Turicibacter*, present in higher proportions in the HFD-CAS
345 group (Table 1), were noted.
346 To assess the possibility that the presence of particular taxa within the chow containing CAS could
347 be responsible for the associated increased proportion of *Streptococcaeae* in the gut, analysis of the
348 microbiota of the CAS-containing chow was performed. This revealed that the family
349 *Streptococcaceae* (99.6% and 99.8% in LFD-CAS and HFD-CAS chow, respectively) and the
350 associated genus, *Lactococcus* (99.6% and 99.8% in LFD-CAS and HFD-CAS chow, respectively),
351 were dominant (Table S2).
352 Correlation and independent analyses of expression association profiles were conducted between
353 *cd36* and *glut2* expression levels, as well as energy intake, against bacterial composition at the
354 genus level. Dairy taxa potentially sourced from the food substrate were not considered. After
355 adjustment for multiple testing (BH-corrected), it was noted that energy intake positively correlated
356 within the group of HFD-CAS samples with *Parabacteroides*. Within the HFD-LAB group,
357 *Parabacteroides* negatively correlated with *cd36* expression. *Glut2* expression was positively
358 correlated with *Parabacteroides* in HFD-LAB samples (Fig. 4C).

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359 **Discussion**

360 In this study, we investigated the effects of a specific dietary whey protein (LAB) on intestinal-
361 adipose-hypothalamic control of energy balance. We show that supplementation of LAB in a HFD
362 reduced *cd36* and *glut2* gene expression in the intestine and was associated with changes in the
363 composition of the gut microbiota. These changes were accompanied by increased cumulative
364 energy ingestion. This, coupled with the fact that targeted deletion of the *glut2* gene increases
365 energy intake[8], suggests a potential link between gut microbiota, intestinal nutrient transporters
366 and energy intake in response to LAB supplementation. These effects were unrelated to adiposity,
367 as HFD-LAB fed mice had similar weight gain, adipose tissue weight (both eWAT and sWAT) and
368 plasma TAG.

369

370 Previous studies have shown that diets rich in whey proteins cause short-term satiety effects in mice
371 and in humans[41; 42; 43]. Long term effects on energy balance, however, do not involve the
372 production of satiety hormones[19]. On a background of HFD intake, which has been shown to
373 reduce the brain sensitivity to satiety hormones[44], the increased energy intake in mice fed with
374 HFD-CAS compared to LFD and even higher (cumulative) energy intake in HFD-LAB is likely to
375 involve another mechanism of regulation within the adipose tissue-gut-brain connection.

376

377 Hypothalamic neuropeptide expression provided an insight into the regulatory effect of LAB on
378 energy intake. Notably, the expression of the anorexigenic neuropeptide *pomc* gene expression was
379 significantly lower in the HFD-CAS group in line with the higher energy intake compared to LFD-
380 CAS, similar to that shown in previous studies[26; 30]. In the HFD-LAB group, while *pomc*
381 expression remained low, the expression of the orexigenic neuropeptide, *npy*, did not change across
382 the groups. In contrast, effects on energy intake and on hypothalamic *pomc* and *npy* expression
383 were previously found in mice fed with WPI compared to casein in a LFD[22]. In addition, neither
384 the expression of *npy* nor the cumulative energy intake changed in mice fed a HFD containing
385 lactoferrin compared to casein[26]. These data suggest that the whey protein LAB have bioactivity
386 that influences energy intake and that is not mediated by the modulation of hypothalamic
387 neuropeptide genes investigated in this study.

388 In the current study, while the HFD-CAS group increased weight rapidly, reaching a significant
389 increase relative to LFD values by week 3, it was a further 3 weeks before the mice fed HFD-LAB
390 diet significantly increased body weight relative to LFD fed animals. The finding that the higher
391 cumulative energy intake was not reflected in the increased body weight in the HFD-LAB group
392 suggests a mechanism must exist to explain the missing energy from the supply. This is consistent

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393 with a rat study, which showed that HFD intake with LAB did not change energy intake but
394 decreased adiposity[27]. The difference in energy intake between this study and our current study
395 could be related to the fact that the rat study included both egg albumin and LAB as the protein
396 sources in the same diet, whereas we used LAB as the only protein source. This apparent energy
397 loss could be related to lipid oxidation, as a recent study showed that pre-exercise LAB-enriched
398 whey protein meal preserved lipid oxidation alongside rapid delivery of amino acids to tissues for
399 use during exercise, decreasing the adiposity in rats[45]. However, we did not observe this effect in
400 the sedentary mice used in our current study, as RER was similar between HFD-LAB and HFD-
401 CAS fed mice and adipose-specific gene expression linked to lipid catabolism also did not change.
402 This, coupled with the similar sWAT and eWAT weights between HFD-LAB and HFD-CAS
403 groups, suggests that the energy loss in the sedentary mice fed LAB occurs through another
404 mechanism.

405

406 It has been shown previously that WPI reduces fat absorption in humans and mice[29; 46; 47], and we
407 showed that this effect could be related to decreased ileal *fatp4* gene expression[22]. In addition, we
408 also showed a time course reduction of *glut2* gene expression in the same region with 17 weeks of
409 WPI intake and this effect on intestinal gene expression was independent of sucrose content in the
410 diet[22]. In the present study, by feeding a constituent protein of WPI, i.e. LAB, to mice for a shorter
411 time period (10 weeks) and by analysing their intestinal gene expression, we now observed a
412 reduction in the expression of *sglt* in both HFD groups relative to LFD groups. This can be a
413 reflection of higher carbohydrate content in the LFD compared to the two HFDs (Table S1). In
414 addition, we show a reduction of *glut2* expression in the ileum exposed to HFD, specifically with
415 LAB. The similar effects of LAB and WPI on intestinal *glut2* expression in the ileum suggests that
416 LAB may contributes to the bioactivity in WPI influencing *glut2* expression and this bioactivity,
417 whatever it may be, appears to be effective independent of sucrose or fat content in the diet[22]. It
418 was shown that the recruitment of apical GLUT2 in the small intestine is detrimental for health, i.e.,
419 it leads to an excess of glucose uptake with increased obesity and type 2 diabetes risk[48]. Our study
420 suggests a potential effect of a diet enriched in LAB in the control of intestinal glucose transporter-
421 related transcripts, that could influence glucose absorption. In another *in vitro* study, using human
422 intestinal Caco-2 cells, it was shown that polyphenols can also lead to a decrease in the abundance
423 of apical GLUT2[49].

424

425 In the current study, we observed also a reduction in *cd36* gene expression within both ileum and
426 jejunum. Thus, in combination, these data further support the hypothesis the whey protein LAB

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427 seems to affect different components of the mechanism involved in the absorption of glucose and
428 fatty acids within the small intestine, at least at the gene expression level. This would explain, at
429 least in part, the loss of energy that occurs in mice fed LAB. Further analysis aimed to look at the
430 protein level and energy content in the faeces need to be done to confirm this hypothesis. Notably,
431 differences in the level of *cd36* and *glut2* in the jejunum during LAB intake are in agreement with
432 the fact that the modality and the degree of absorption can change along the intestinal tract. This is
433 due to a different density of nutrient transporters across the three major regions of the small
434 intestine[(50)]. Based on the effect of LAB in a HFD on expression of nutrient transporters in the
435 intestinal epithelium and associated energy loss, we speculate that mice compensate by increasing
436 energy intake. In fact, in this study we observed that the levels of glucose and triacylglycerol in the
437 plasma did not show significant changes between HFD groups.
438
439 Furthermore, the HFD groups consumed more energy than LFD. Since there were no differences in
440 the energy expenditure, the energy surplus has been stored in the adipose tissue. Thus, the lower
441 expression of *acc* and *fasn* in the adipose tissue, presumably reflects the reduced necessity to
442 produce fat endogenously when there is an external supply of excess energy. A similar reduction in
443 *fasn* gene expression has been noted in a previous study from our group[(21)]. On the contrary, other
444 studies show that HFD intake increases *fasn* and *acc* in the adipose tissue[(51; 52; 53)]. The reasons for
445 these discrepancies are unclear, but we speculate that contributing factors can include different
446 genetic background, age, diet (composition and duration) and different housing environment.
447
448 Leptin, a hormone encoded by the *ob* gene, controls food intake and body weight through an
449 interaction with specific receptors within the hypothalamus[(54)]. In obese humans and in mice fed an
450 obesogenic diet the adipose tissue mass increases, enhancing significantly the level of serum leptin
451 and leading to metabolic dysfunctions[(55)]. In addition to the effect on intestinal nutrient transporters
452 and energy intake, WPI in a LFD also reduced eWAT weight and plasma leptin, raising the
453 possibility that the reduced adipose tissue and associated signals, including reduced leptin, could
454 have stimulated the energy intake in mice. However, feeding mice with a HFD containing 20% of
455 WPI for 8 weeks did not have an effect on plasma leptin levels, compared to mice fed a HFD
456 containing CAS[(30)]. On the contrary, HFD containing lactoferrin significantly reduced plasma leptin
457 levels compared to the HFD-CAS controls[(26)]. This is another demonstration of the distinct
458 bioactivities that WPI-associated whey proteins can have. In this study, mice fed a HFD containing
459 LAB as the sole protein source showed no significant decrease in plasma leptin, which was

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460 reflected in no change in eWAT and sWAT *ob* gene expression in the HFD-LAB group, compared
461 to the HFD-CAS group.
462
463 We also demonstrated that a HFD containing LAB has a strong influence on the composition of the
464 gut microbiota. Beta-diversity data highlighted differences in the clustering of samples from the
465 HFD-LAB group compared to the HFD-CAS group. Corresponding taxonomic analyses were also
466 completed. In particular, we observed a significantly higher proportion of *Parabacteroides* in the
467 HFD-LAB group, which is notable in that this genus showed a significantly positive correlation
468 with the expression of nutrient transporters. Notably other studies have suggested that
469 *Parabacteroides* protects the gut from inflammation and there have been reports of the apparent
470 absence of this genus in the digestive tract of people affected by Inflammatory Bowel Diseases[56;
471 57]. We also observed that the genera *Bifidobacterium* and *Lactobacillus* are significantly more
472 abundant in the HFD-LAB group. In several human studies, an association between lower levels of
473 *Bifidobacterium* and obesity was noted[58; 59; 60]. In addition, higher levels of *Bifidobacterium* have
474 been linked with an increase in SCFA production, an improvement of gut mucosal barrier and lower
475 intestinal LPS levels[60; 61]. Furthermore, specific strains of *Bifidobacterium* and *Lactobacillus* spp.
476 have been shown to exhibit anti-obesity effects[62]. It is worth highlighting the importance of species
477 and strain level differences as, for example, human and animal studies have highlighted an
478 association between different *Lactobacillus* species with weight gain or weight protection[63; 64]. For
479 this reason, more accurate in-depth metagenomic analyses, such as shotgun analysis, should be
480 considered in the future to investigate the *Lactobacillus* species that populate the gut of HFD-LAB
481 fed mice.
482 A number of bacterial taxa typically associated with dairy products were also found to be altered in
483 the gut. A recent human feeding study demonstrated that many bacteriophages and *Streptococcus*
484 and *Lactococcus* genera, that were significantly altered in individuals whose diet was supplemented
485 with whey proteins, were present in high proportions within the whey protein supplement[65], and
486 thus presumably originated from the food source. The family *Streptococcaceae* and the associated
487 genus, *Lactococcus*, are significantly more abundant in the HFD-CAS group compared to the HFD-
488 LAB group. This is consistent with our previous study that showed that *Streptococcaceae*
489 proportion increased significantly in the CAS-fed mice compared to WPI-fed mice[22]. However,
490 this phenomenon is likely a reflection of the high proportion of *Streptococcaceae* and *Lactococcus*
491 reads detected after analysis of both LFD-CAS and HFD-CAS chows.
492 The changes in the gut microbiota in the presence of LAB, coupled with the reduced intestinal
493 expression of genes for nutrient transporters, support and provide a potential scenario for the energy

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494 mismatch in the HFD-LAB group compared to the control group. However, the specific mechanism
495 that connects these changes in intestinal transporters gene expression, gut microbiota and energy
496 intake in the presence of LAB remains to be elucidated. Further studies are needed to assess if these
497 effects are seen with different genetic background, sex, housing environment (group versus single
498 housing) and dietary fat content and duration. In conclusion, this present study has demonstrated for
499 what is to our knowledge the first time, how a HFD containing a specific whey protein, i.e. LAB,
500 specifically affects the metabolism, microbiota and nutrient absorption regulation, without
501 preventing weight gain and adipose tissue mass accumulation in mice.
502
503
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513 **Conflict of Interest**
514 None.
515
516 **Authorship**
517 The author contributions are as follows: K. N. N., P. D. C., J. R. S. and J. F. C. designed the study;
518 S. B. and K.N.N. performed the experiments; R. C. R. carried out the bioinformatics analyses; S.B.,
519 K.N.N. and R.C.R generated the figures. All authors contributed to the drafting of the manuscript.
520 All the authors approved the final version for submission.
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535 **Table 1**
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537

Family	HFD-LAB vs HFD-CAS	Relative Abundance
Streptococcaceae	P=0.029 ↑ HFD-CAS	0.082895775
Lactobacillaceae	P=0.015 ↑ HFD-LAB	1.18133309
Genus		
Lactococcus	P=0.001 ↑ HFD-CAS	0.077810661
Turicibacter	P=0.024 ↑ HFD-CAS	0.301261529
Streptococcus	P=0.001 ↑ HFD-LAB	0.148479572
Parvibacter	P=0.039 ↑ HFD-LAB	0.012092907
Lactobacillus	P=0.011 ↑ HFD-LAB	1.180503082
Parabacteroides	P=0.026 ↑ HFD-LAB	0.045986668
Bifidobacterium	P=0.05 ↑ HFD-LAB	0.159330093

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548 **Table 1:** Relative abundance (%) of genera and families in which their abundance is significantly
549 higher either in the HFD-CAS group or in the HFD-LAB group. Statistical differences across
550 multiple samples were estimated by the bioinformatics methods "False discovery rate" control
551 based on the "Benjamini-Hochberg" procedure
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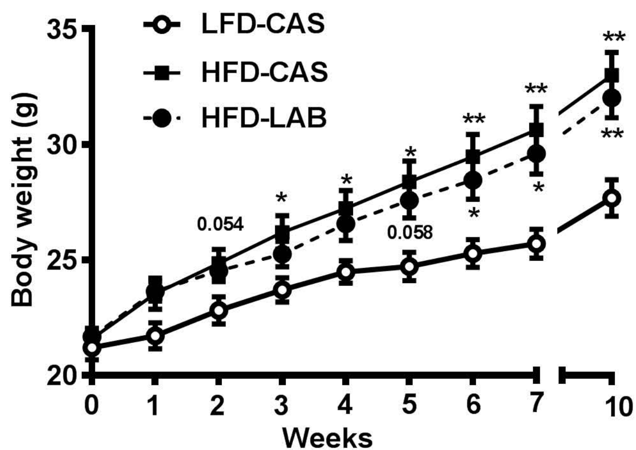

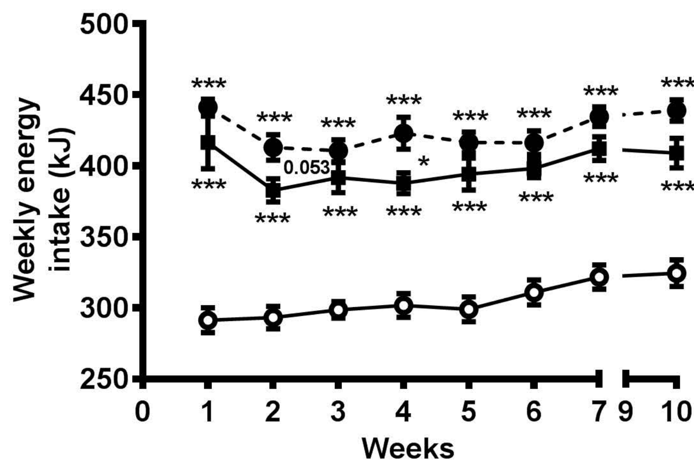

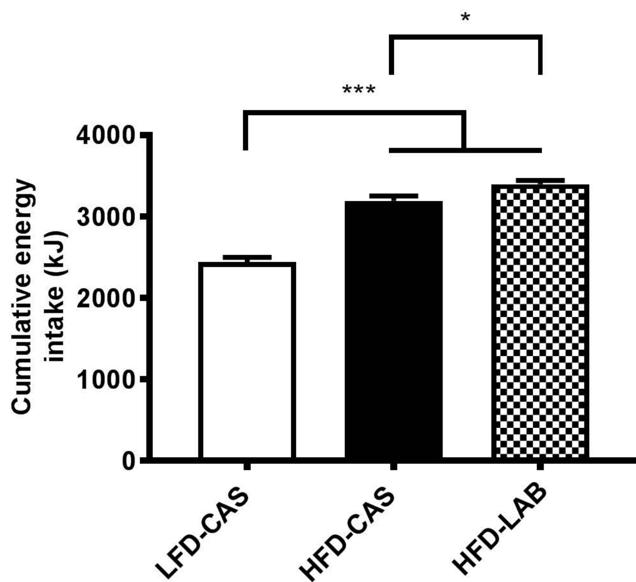

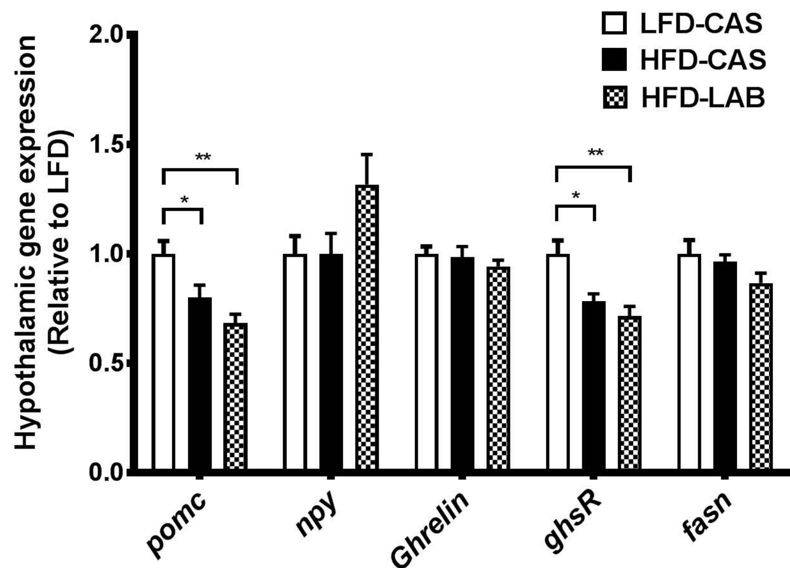

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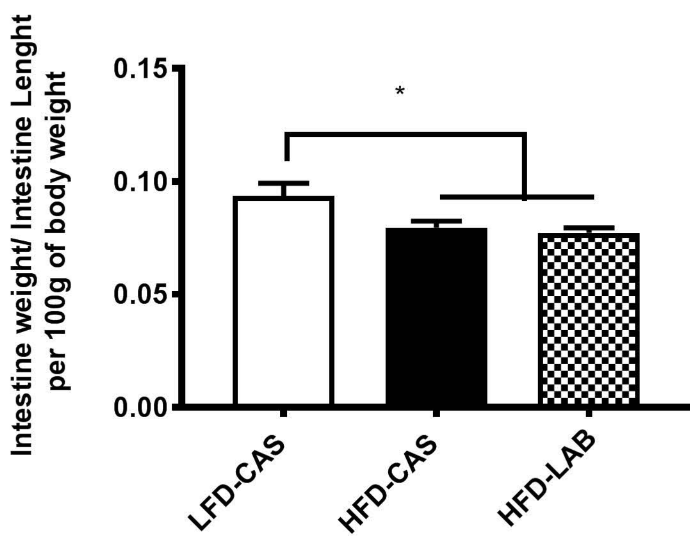

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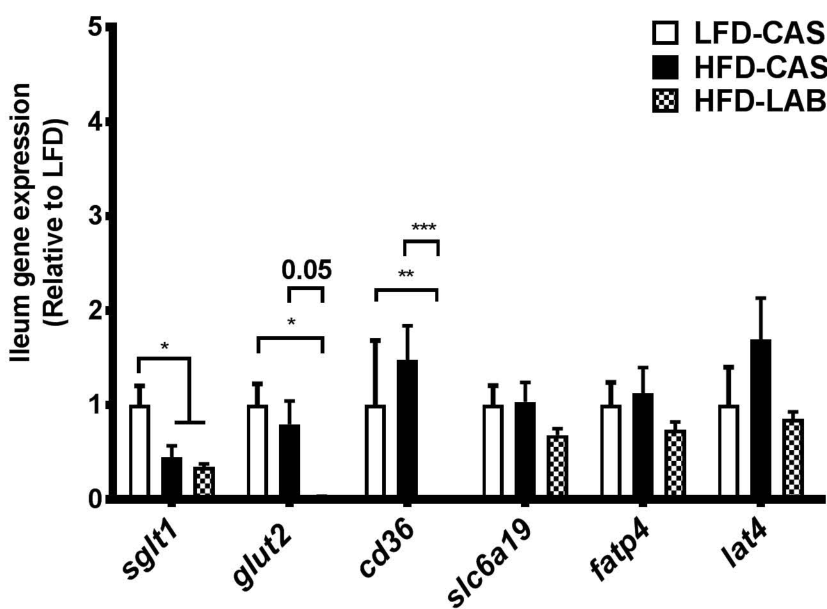

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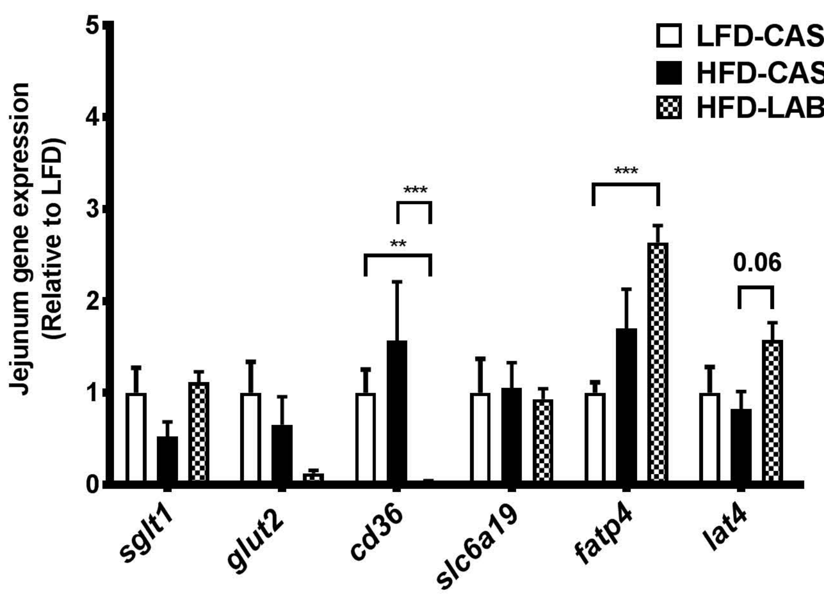

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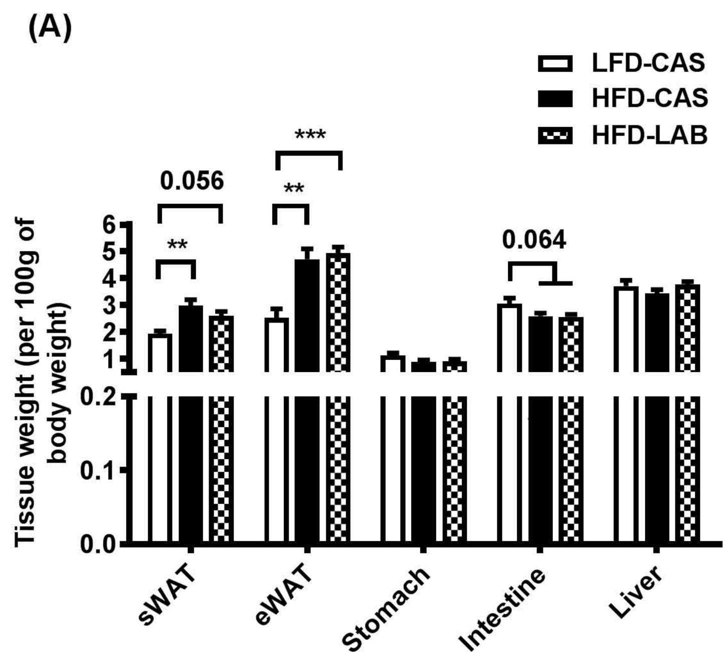

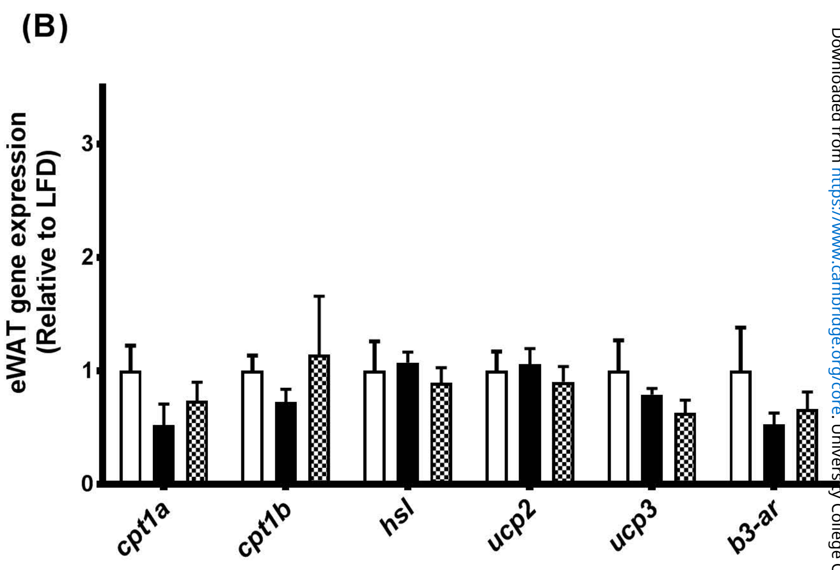

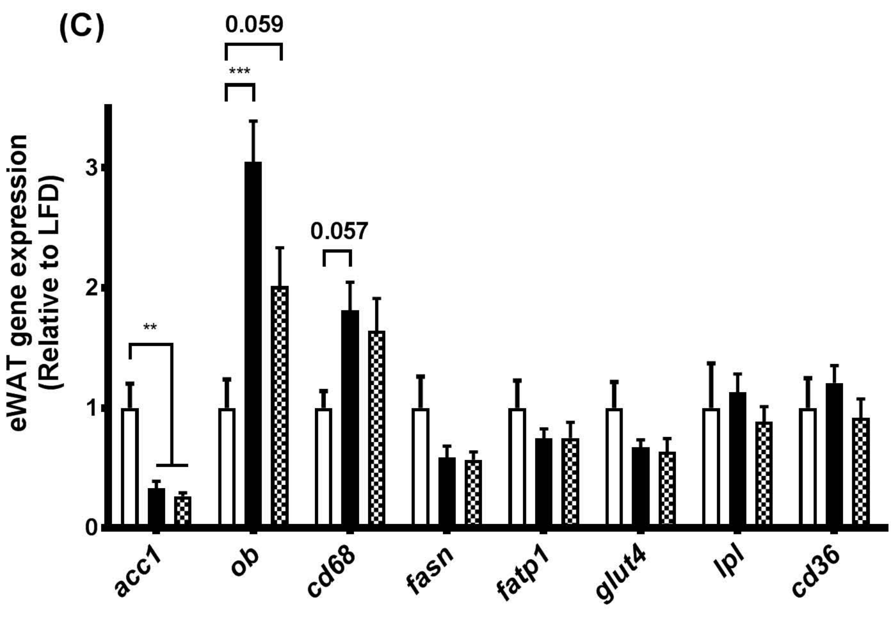

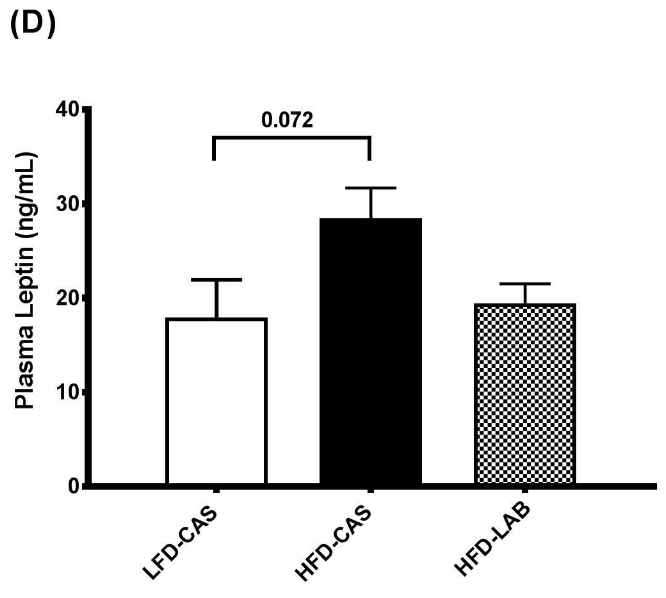

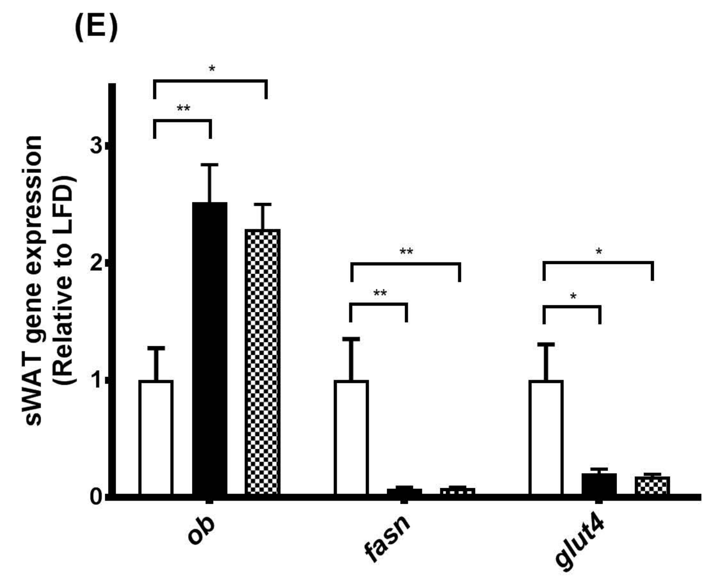

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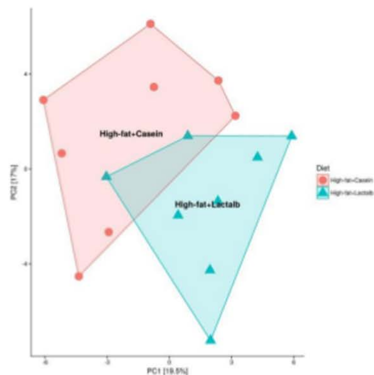

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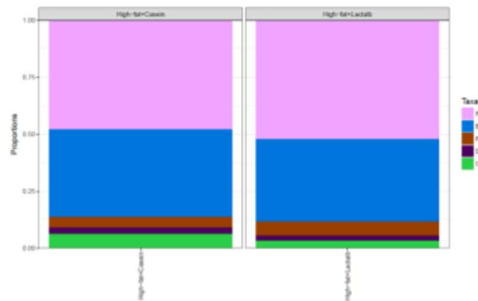

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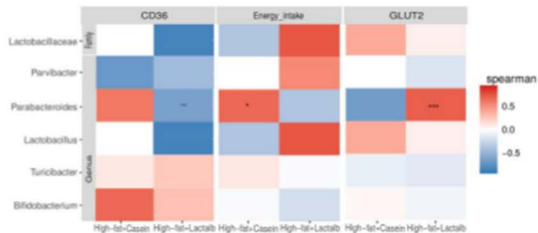

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