TITLE: Whey protein isolate decreases murine stomach weight and intestinal length and alters the expression of Wnt signalling-associated genes.

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Whey protein isolate decreases murine stomach weight and intestinal length and alters the expression of Wnt signalling associated genes

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Running Title: Whey proteins influence gastro-intestine

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Abstract

This study examined the underlying mechanisms by which whey protein isolate (WPI) affects energy balance. C57BL/6J mice were fed a diet containing 10% kJ fat, 70%kJ carbohydrate (35%kJ sucrose) and 20% kJ casein or WPI for 15 weeks. WPI reduced weight gain, cumulative energy intake and dark phase oxygen consumption $_{(V02)}$ compared to casein fed mice ($P<0.05$), but had no significant effects on body composition, meal size/number, water intake or respiratory exchange ratio (RER). Plasma levels of insulin, triacylglycerides (TAG), leptin, glucose, and glucagon-like peptide 1 remained unchanged. Notably, WPI reduced stomach weight and both length and weight of the small intestine ($P<0.05$). WPI reduced gastric expression of Wnt5a ($P<0.01$) and Frizzled 4 (Fzd4) ($P<0.01$) with no change in expression of receptor tyrosine kinase-like orphan receptor 2 (Ror2) and low density lipoprotein receptor-related protein 5 (Lrp5). In the ileum, WPI increased Wnt5a mRNA expression ($P<0.01$) and caused a trend towards increased expression of Fzd4 ($P=0.094$), with no change in Ror2 and Lrp5. These genes were unresponsive in the duodenum. Among nutrient responsive genes, WPI specifically reduced ileal mRNA expression of peptide YY ($P<0.01$) and fatty acid transporter 4 ($P<0.05$), and decreased duodenal mRNA expression of the insulin receptor ($P=0.05$), with a trend towards a decreased expression of sodium-glucose co-transporter 1 ($P=0.07$). The effects of WPI on intestinal Wnt signalling may explain how WPI affects gastrointestinal structure and function and in turn energy intake and balance.

Introduction

Whey proteins are a by-product of cheese manufacture. These dietary proteins have gained considerable interest in recent years in relation to their health benefits, which include reduction in energy intake and body weight, and improvements to insulin sensitivity [1; 2]. However, more research is required to assess if whey protein effects are dependent upon macronutrient composition in the diet, as demonstrated previously for high whey protein enriched diets[3], as this will allow creation of diets with the optimum macronutrient combination that enhances specific whey protein effects on human health. Additionally, this work may also uncover the mechanisms underpinning the effects of these proteins.

We have previously shown that intake of 20% energy whey protein isolate (WPI) reduces weight gain, fat mass and increases lean mass in mice fed a high fat (45% by energy) diet[4; 5]. In contrast, on a low fat diet (e.g. 10% fat by energy), an impact on energy balance has been observed when a high whey protein content was used in the test diets, where the protein derived energy content was far beyond the adequate range (14-25%). Moreover, where effects were found, the underlying mechanisms have not been elucidated. For instance, mice fed a diet containing 30% energy from whey proteins and 10% energy from fat reduced energy intake and weight gain by 1 week compared to similar diet but with either soy or gluten as the protein source[6]. A reduction in cumulative energy intake was observed in rats between 5 to 10 weeks when fed a diet providing 42% energy from proteins (26% whey and 16% albumin) in comparison to a similar diet but with the whey replaced by either albumin or soy protein with albumin[7]. In contrast, when the dietary protein derived energy content was reduced to 15 or 25%, with fat derived energy content was maintained around 9 and 15%, respectively, whey proteins did not affect energy intake and body weight gain[8; 9]. Interestingly, the latter studies assessed food intake only for 2 or 7 week duration, in comparison to the high whey protein study, where the reduction in energy intake occurred by week 5 and continued up to 10 weeks[7]. Thus, the discrepancy in the data could be due to the test duration, where a longer duration is required when adequate protein derived energy content is used in test diets. Thus, the purpose of this study was to assess if WPI at 20% energy content in a 10% energy fat diet alters energy balance and where there is an effect, to explore the mechanisms involved. The study lasted for 15 weeks in consideration the previous published data showing that whey protein (at adequate range) did not affect energy balance up to 7 weeks[8; 9].

Materials & Methods

Animals & diets

All procedures involving animals were approved by the University College Cork Animal Experimentation Ethics Committee (~201 1/005) and were licensed under the Cruelty to Animals Act 1876. Three week old C57BL/6J male mice (Harlan; UK) were singly housed and had *ad libitum* access to food and water throughout the trial. Mice were initially fed a diet containing 10%kJ fat, 70%kJ carbohydrate (35%kJ sucrose) and 20%kJ casein (LF-CAS; #D12450; Research Diets, USA) during the acclimatisation period lasting three weeks.

Experimental protocol

Following acclimatisation, singly housed mice were separated into two weight matched dietary groups and provided either the LF-CAS diet or a comparable LF with WPI (Alacen^{tm} 895 NZMP, Auckland, New Zealand) (LF-WPI) for a total of 15 weeks (n = 8). Further details of the diet composition can be found in the supplementary table 1. Body weight and energy intake were measured weekly. In addition, at week 14, feeding behaviour and metabolic activity were measured using TSE Phenomaster system compromising 8 test cages and one reference cage (Bad Homburg, Germany). Mice were housed individually in the test cages. After a 2 day acclimatisation period, metabolic parameters were measured on the 3rd day. Sensors recorded metabolic parameters in cages sequentially with each cage being analysed for one minute, such that data collected over 24h period corresponded to 9 minute intervals for the 8 test cages and one reference cage. Body composition was determined by NMR using a Bruker minispec LF50H (Bruker optics, Germany) at the end of the study, following a 6-8h fast. Subsequently, mice were anesthetised and blood was collected. The mice were then sacrificed by cervical dislocation. Tissues of interest were excised and weighed. Small intestinal luminal contents were removed by mechanical expulsion and the tissue length was determined. The tissue was weighed and 2cm sections from the proximal and distal parts of the intestine that were 2cm away from the start and end of the intestine, respectively, were excised for analysis of gene expression in the duodenum and ileum. All tissues were immediately snap frozen in liquid nitrogen. Plasma and tissue samples were stored at -80^{o}C until analysis.

Biochemical analysis

Blood collected into vacutinater EDTA tubes (BD; USA) from anesthetised mice (ketamine; 65mg/kg, xylazine; 1 3mg/kg), was treated with Diprotin A and Aprotinin as described previously (4) to protect plasma peptides against proteolytic degradation. Plasma was isolated by centrifugation at 2000rpm and 4^{o}C for 15 mins, and was analysed using colorimetric assay kits to determine plasma concentrations of glucose (Calibochem, Germany) and TAG (LabAssay TAG, Wako Chemicals,

136 USA). ELISA kits were used determine plasma concentrations of glucagon-like peptide 1 (GLP-1)
137 (Millipore, USA), leptin and insulin (Crystal Chem, USA). Homeostasis model assessment of
138 insulin resistance (HOMA-IR) was calculated as previously detailed[4].
139
140 **Tissue gene expression**
141 Total RNA was isolated from tissues of interest (epididymal adipose tissue, liver, skeletal muscle,
142 duodenum and ileum) using RNeasy mini kits (Qiagen, Germany) with subsequent DNase treatment
143 to prevent genomic DNA contamination. Duodenal and ileal total RNA was isolated from
144 representative intact tissue segments. Complementary DNA was synthesised from 1µg of isolated
145 total RNA and subjected to Real-time qPCR analysis as described previously[4]. Primer sequences
146 of target and reference genes are listed in supplementary table 2. Target gene mRNA expression
147 was calculated using $^{2\text{-}\Delta\Delta Cp,}$ after normalising against the reference gene expression according to
148 ΔΔCp= ΔCp target gene – ΔCp reference gene. Reference genes used were β-actin (adipose, liver,
149 muscle, duodenum, ileum), YWHAZ (liver and duodenum, ileum) and 18-S (adipose). In instances
150 where two housekeeping genes were used, a geometric mean was determined for normalisation. The
151 gene expression is shown relative to the LF-CAS group.
152
153 **Statistical analysis**
154 The data are presented as mean with standard error (SEM). Dietary treatment effects were analysed
155 using unpaired T-tests. A two-way Repeated measures ANOVA with *Bonferroni* multiple
156 comparisons was used to analyse bodyweight trajectories over the treatment periods. Mann-
157 Whitney U tests were used to analyse non-parametric data. VO_2 data were analysed by ANCOVA
158 (SAS software version 9.3, USA) using total body weight as the co-variant as detailed
159 previously[10]. Statistical significant was established at $P \leq 0.05$, using Graphpad Prism (ver. 6),
160 SAS software version 9.3, USA) and Minitab (ver. 15).
161
162 **Results**
163
164 **Effect on weight gain and body composition**
165 Cumulative energy intake was significantly reduced in WPI fed mice over the first 13 weeks, and
166 this was significant by week 11 onwards (Figure 1D; $P < 0.05$). In contrast, body weight gain was
167 not significantly different at week 11 (6.17±0.28g in LF-WPI versus 7.05±0.54g in LF-CAS;
168 P=0.17), but reached significance by week 12 (5.89±0.24g in LF-WPI versus 7.5±0.61g in LF-CAS;
169 P<0.05). Although after 15 weeks of dietary intake, total body weight gain was significantly

reduced in the LF-WPI group compared to LF-CAS group (Figure 1B; $P < 0.05$), at this time point, there was no significant difference in either body weight or body composition (fat or lean mass %) between the two groups (Figure 1A and C). In correspondence with overall % body fat mass, the mass of both the subcutaneous and epididymal adipose tissue depots was not different between the LF-CAS and LF-WPI fed mice (Figure 2A).

Effect on energy intake and metabolism related parameters

Further analysis of energy intake using TSE Phenomaster cages in week 14 (Table 2), showed a reduction in the dark-phase energy intake in the WPI group, although this was not significant. There were also no significant effects on the 24hr meal pattern, represented by meal number and size, RER and water intake but dark phase $_{VO2}$ decreased by WPI and there was also a trend towards a decrease in $_{VO2}$ during light phase (Table 2), which was revealed by analysis of metabolic data independent of body weights of the animals.

Effect on the gastrointestinal tract

Mice fed WPI had reduced stomach and small intestine weights relative to casein controls after 15 weeks of dietary treatment, which also corresponded with a concomitant reduction in small intestinal length (Figure 2).

At a cellular level, WPI intake reduced gene expression of Wnt5a and Frizzled 4 (Fzd4) in the stomach but not the expression of receptor tyrosine kinase-like orphan receptor 2 (Ror2) and low density lipoprotein receptor-related protein 5 (Lrp5) (Figure 3A). In the ileum, WPI increased Wnt5a mRNA expression and caused a trend towards increased expression of Fzd4 (P=0.094)(Figure 3C). The Wnt signalling associated genes in the duodenum did not respond to WPI (Figure 3B). However, in the same region, WPI intake caused a trend towards a decrease in gene expression of insulin receptor (IR) and sodium-glucose co-transporter 1 (SGLT-1)(Table 3) with no change in insulin receptor substrate 1 (IRS 1), glucose transporter 2 (GLUT2) or glucose-dependent insulinotrophic peptide (GIP)(Table 3). Cholecystokinin (CCK) gene expression was decreased by WPI but not significantly (Table 3). In the ileum, GLUT2 gene expression was increased by WPI, whilst fatty acid transporter 4 (FATP4) expression was decreased (Table 3). Ileal proglucagon expression was unaffected by WPI (Table 3), which correlated with plasma GLP-1 level (Table 1), but PYY gene expression was reduced by WPI (Table 3).

Investigation of the same tissues in a previous published study conducted in mice fed a similar LF-casein and LF-WPI diet for 7 weeks[5], revealed that whilst the stomach weight was not different between the two groups (1.06 ± 0.10 in LF-WPI versus 1.00 ± 0.048 in LF-CAS; data relative to

204 LF-CAS set at 1.00), the intestinal weight in WPI fed mice was reduced relative to casein fed
205 controls (0.73 ± 0.026 in LF-WPI versus 1.00 ± 0.035 in LF-CAS; $P < 0.001$; data relative to LF-
206 CAS set at 1.00), as was the intestinal length (33.65 ± 0.38 cm in LF-WPI versus 35.64 ± 0.70 cm
207 in LF-CAS; $P < 0.05$).
208
209 **Effect on plasma hormones and metabolites and lipid metabolism related gene expression**
210 Although WPI did not affect plasma leptin (Table 1) and related epididymal gene expression
211 (Figure 4), or plasma levels of TAG, insulin and glucose (Table 1), epididymal expression of fatty
212 acid synthase (FASN), acetyl CoA carboxylase (ACC) and cluster of differentiation 36 (CD36)
213 were significantly up-regulated in WPI fed mice compared to controls at 15 weeks (Figure 4).
214 Additionally, lipoprotein lipase (LPL) gene expression also showed a trend towards an increase (P
215 = 0.08)(Figure 4). Genes involved in insulin signalling (IR, GLUT4 and IRS-1), fatty acid
216 catabolism (β3-AR, HSL, CPT1b and UCP-2) and adipogenesis (PPARγ) were unaltered by WPI
217 intake (Figure 4). None of the insulin signalling or lipid metabolism related genes were altered by
218 WPI intake in the muscle or liver (Supplementary table 3 and 4) with the exception of IRS-1, which
219 showed a trend towards a decrease in the muscle in LF-WPI fed mice compared to controls
220 (Supplementary table 3).
221
222 **Discussion**
223 Our results showed that 15 weeks of WPI intake decreased weight gain, cumulative energy
224 intake and dark phase metabolic activity (V_{02}), which was accompanied by a reduction in stomach
225 weight and intestinal length and weight in mice fed a low fat diet. WPI fed mice reduced cumulative
226 energy intake by week 11. The dark phase metabolic activity, which was measured at week 14, also
227 decreased in WPI fed mice compared to casein controls. The reduced body weight gain with WPI
228 intake could thus be a consequence of the gradual decrease in cumulative energy intake occurring
229 before the change in metabolic rate. Intestinal satiation/satiety-related CCK and proglucogon genes
230 were unresponsive to the WPI intake, as was plasma GLP-1 level. Moreover, ileal expression of
231 PYY, which reduces energy intake[11], was significantly decreased in LF-WPI fed mice compared to
232 LF-fed casein controls. These data suggest that modulation of the production of these satiety and
233 satiation hormones was not the mechanism by which WPI reduces cumulative energy intake.
234 The current data show that WPI intake reduces intestinal length. Given the association
235 between reduction in the intestinal length and weight loss [12], here we sought to obtain further
236 evidence supporting a link between WPI-induced changes in intestinal cellular activity and energy
237 balance in mice by investigating nutrient responsive genes in the duodenum and ileum. Consistent

with a reduced nutrient contact with or passage through the intestinal cells, duodenum expression of IR and SLGT-1, that is responsive to glucose[13], showed a trend towards a decrease in WPI fed mice compared casein controls. In the ileum, the cellular lipid transport protein FATP4[14], and PYY, which are mainly responsive to availability of fat[15], were both significantly decreased by WPI intake. Although duodenum mRNA expression of GLUT2, the protein product of which is predominantly found at the basolateral membrane of enterocytes[16], was increased by WPI intake, one could argue based on other nutrient responsive genes that this change may represent a feedback mechanism to the lower availability of nutrients in the luminal side of the intestine. The gene expression data in the intestine are consistent with the changes in cumulative energy intake in the animals.

To ascertain the timeframe of the change in intestine length and weight relative to energy intake, we investigated the same tissues in an earlier published study conducted in mice fed the same LF-CAS and LF-WPI diets, but where the study was terminated at week 7[5] . In that study, by week 7, mice fed with WPI consumed a similar energy content to their casein counterparts, and had reduced intestinal weights and lengths similar to the present study. This suggests therefore that the reduction in cumulative energy intake, which reached significance compared to casein fed mice by week 11, occurred after the morphological change in the intestine. It is noteworthy that obese *ob/ob* mice have longer intestines[17] and that the development of high fat diet-induced obesity in mice also increases villus lengths and cells per villus[18]. Moreover, recent study showed that WPI intake reduced growth-related parameters within the first week of diet intake[19]. The current data also show that WPI intake reduced stomach weight in mice. In contrast, in our previous published 7 week study, mice fed with WPI had an unaltered stomach weight, suggesting that the reduction in stomach weight occurred between 7 to 15 weeks of WPI intake, after the reduction in intestinal length. These data together suggests a potential functional relationship between WPI induced changes in gastro-intestinal tract and energy intake.

Organ development is in part regulated by the Wnt/β-catenin pathway involving Wnt proteins, Fzd and Lrp 5/6 receptor complexes, which interact and mediate effects through the β-catenin proteins[20]. Notably, Wnt5a has been shown to act independent of β-catenin pathway (the noncanonical signalling) but recent data suggest that it can also inhibit and stimulate the canonical Wnt/β-catenin by acting through the Ror2 receptor, which has been revealed by generation of transgenic mice over-expressing Wnt5a[21] and by overexpression of Fzd4 and Lrp5 in 293cells, where the experimental manipulation allowed Wnt5a to stimulate Wnt/β-catenin signalling[22]. Notably, mice ectopically expressing Wnt5a were stillborn and had reduced stomach and intestinal tracts[21]. Interestingly, targeted deletion of the Wnt5a gene in mice also caused changes in the

gastrointestinal tract[23] similar to mice over-expressing the same gene[21]. Contrasting genetic manipulations of Wnt5a resulting in similar gastro-intestinal features in mice suggest a complex interplay between Wnt5a noncanonical signalling pathway and Wnt/β-catenin pathway. This complexity is further highlighted in the present study, as Wnt5a and Fzd4 gene expression decreased in the stomach in WPI fed mice compared to the controls, whilst the same genes in the ileum responded to the protein intake by increasing their expression, yet the two tissues reduced in weight. In contrast, neither were affected by WPI in the duodenum, with Ror2 and Lrp5 remaining unchanged throughout the gastro-intestinal tract where the gene expression was investigated. Importantly, this study demonstrates that Wnt signalling associated genes in the gastro-intestinal tract are responsive to WPI. We speculate that the regional specific expression and actions of Wnt5a and Fzd4 and associated inhibitory or stimulatory effects on the Wnt/β-catenin signalling pathway underlie the observed changes in the gastro-intestinal tract of WPI fed mice, which in turn modulate the energy intake and body weight gain.

Although whey protein intake has been shown to cause an insulinotropic effect[24; 25], in the current study, WPI did not alter plasma levels of insulin, glucose or GLP-1, intestinal expression of GIP or insulin signalling associated genes in the liver, muscle or epididymal tissues. The lack of an insulinotropic effect could be due to the reduction in cumulative energy intake and associated reduced nutrient availability and/or due to the high sucrose content in the diet, which has previously been shown to cause glucose intolerance and impair GLP-1 secretion in mice[26]. Notably, IR expression in the duodenum showed a trend towards a decrease in response to WPI intake. Whether this related to the altered intestinal morphology remains to be determined because insulin action in the intestine has been shown to promote greater enterocyte turnover, which correlates with insulin-receptor expression in the villus-crypt axis[27].

In the current study there was a mismatch in the time frame of decreased cumulative energy intake and body weight gain as the former decreased by week 11, whilst the body weight gain was significantly different by week 12. Even at the end of the study, the plasma TAG level was not different between the two groups of mice, nor was percentage fat content in the body or specifically in the epididymal and subcutaneous fat pads. It is noteworthy in this respect that the epididymal expression of lipogenic ACC and FASN increased in association with a concurrent increased fatty acid transporter CD36 expression[28]. These data suggest an increased lipogenesis in the epididymal adipose tissue, which may be acting to maintain energy homeostasis causing a mismatch in the time frame between a decreased cumulative energy intake and body weight. In line with this suggestion, metabolic activity, which was measured at week 14, also decreased by WPI intake, presumably because of decreasing cumulative energy in mice with altered gastro-intestinal tracts. In contrast, in

306 our previous published studies, WPI at 20% energy content within a 45% energy fat diet reduced fat
307 mass and normalised energy intake without affecting metabolic activity in mice at 8 and 21 weeks
308 compared to casein fed control[4; 5] . In the latter studies, data for metabolic activity was shown
309 relative to body weight (ml/hr/kg). In light of the suggestion that ANCOVA is a more suitable
310 statistical method to identify effects of diet on metabolic activity independent of body weight
311 changes, we re-analysed these previously published data using ANCOVA[10]. Again, as our
312 previous published Vo_2 data (in ml/hr/kg)[4], there was not significant effect of WPI on Vo_2 (ml/hr)
313 independent of the body weight in the mice fed a high fat diet with WPI (data not shown). Thus,
314 contrasting WPI effects on energy intake, fat mass and metabolic activity in the previously
315 published studies[4; 5] compared to the present study could be related to how whey proteins affect
316 energy balance in a background of altered fat to carbohydrate content in the diet.
317 In conclusion, the results here show that 20% energy WPI in a 10% fat and 35% sucrose diet
318 reduced cumulative energy intake, dark phase metabolic activity and body weight gain. This is
319 accompanied by a decreased intestinal length and weight, as well as stomach weight, which was
320 correlated with altered expression of Wnt signalling associated genes. Further investigations are
321 required to determine the significance of the alteration to the intestinal environment uncovered here
322 for energy balance regulation.
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Conflict of interest

Authors declare that there is no completing interest.

Authors' contribution

LM, JRS, JFC and KNN designed the study, KN and JFC obtained ethical approval for the study, LM performed the experiments. LM and JRC analysed the data and LM generated the figures. All authors contributed to drafting of the manuscript. All authors approved the final version for submission.

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448

449 **Figure legends**

450 **Figure 1. Effect of whey protein isolate on energy balance related parameters.** Mice were fed a
451 10% kJ fat and 35%kJ sucrose diet with either 20% kJ casein (LF-CAS) or whey protein isolate
452 (LF-WPI) for 15 weeks. The body weight trajectories over the 15 weeks are shown (A), together
453 with weight gain (B), body composition (C) and cumulative energy intake over the first 13 weeks
454 (D). Data represent means ± SEM (n = 8). * $P < 0.05$, ** $P < 0.01$.
455

456 **Figure 2. Effect of whey protein isolate on tissue weights (A) and intestinal length (B).** Mice
457 fed a 10% kJ fat and 35%kJ sucrose diet with either 20% kJ casein (LF-CAS) or whey protein
458 isolate (LF-WPI) for 15 weeks. Tissue weights are shown relative to casein fed animals. SAT,
459 subcutaneous white adipose tissue; EAT, epididymal white adipose tissue. Data represent means ±
460 SEM (n = 8). * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.
461

462 **Figure 3: Effect of whey protein isolate on gastro-intestinal Wnt gene expression.** Mice were
463 fed a 10% kJ fat and 35%kJ sucrose diet with either 20% kJ casein (LF-CAS) or whey protein
464 isolate (LF-WPI) for 15 weeks, and the mRNA expression were measured in (A) stomach, (B)
465 duodenum and (C) ileum. Gene expressions are shown relative to the LF-CAS group which was set
466 at 1.00. Fzd4, Frizzled 4; Ror2, receptor tyrosine kinase-like orphan receptor 2; Lrp5, low density
467 lipoprotein receptor-related protein 5. Data represent means ± SEM (n = 6-8). * $P < 0.05$, ** $P <$
468 0.01.
469

470 **Figure 4. Effect of whey protein isolate on epididymal adipose gene expression.** Mice were fed
471 a 10% kJ fat and 35%kJ sucrose diet with either 20% kJ casein (LF-CAS) or whey protein isolate
472 (LF-WPI) for 15 weeks, and the mRNA expressions of epididymal adipose tissue genes were
473 measured. Gene expressions are shown relative to the LF-CAS group which was set at 1.00. Genes
474 investigated were insulin receptor (IR), glucose transporter 4, (GLUT4), insulin substrate 1 (IRS-1),
475 βeta-3 adrenergic receptor (β3-AR), hormone sensitive lipase (HSL), carnitine palmitoyltransferase
476 1b, (CPT1b), uncoupling protein 2 (UCP-2), peroxisome proliferator-activated receptor gamma
477 (PPARγ), lipoprotein lipase (LPL), fatty acid transporter 1 (FATP1), cluster of differentiation 36
478 (CD36), fatty acid synthase (FASN), and acetyl-CoA carboxylase (ACC). Data represent means ±
479 SEM (n = 8). * $P < 0.05$, * * $P < 0.01$.
480

Table 1

Plasma parameters in mice fed a LF-CAS or LF-WPI diet for 15 weeks.

	LF-CAS	SEM	LF-WPI	SEM	*P* value
Plasma TAG (mg/dL)	36.18	1.42	33.68	2.59	NS
Plasma leptin (ng/ml)	6.65	1.53	5.80	1.38	NS
Plasma GLP-1 (pM)	24.70	3.50	23.59	1.50	NS
Plasma glucose (mmol/L)	12.18	1.57	13.11	0.84	NS
Plasma insulin (ng/ml)	0.21	0.01	0.22	0.01	NS
HOMA-IR	3.07	0.39	3.12	0.16	NS

Data are means ± SEM (n = 8). LF-CAS, 10%kJ fat and 35%kJ sucrose with 20%kJ casein; LF-WPI, 10%kJ fat and 35%kJ sucrose with 20%kJ whey protein isolate (WPI). GLP-1, glucagon-like peptide 1; HOMA-IR, homeostasis model assessment of insulin resistance; NS, not significant.

488 **Table 2**

489 Metabolic parameters in mice fed a LF-CAS or LF-WPI diet at week 14.

	Phase	LF-CAS	SEM	LF-WPI	SEM	*P* value
Energy intake (kJ/mouse)	Light	12.17	1.46	10.40	2.42	NS
	Dark	43.06	2.10	32.86	3.91	NS
Meal number	Light	13.0	1.1	20.3	5.0	NS
	Dark	31.9	2.9	25.4	3.0	NS
Meal size (kJ)	Light	0.99	0.16	0.80	0.12	NS
	Dark	1.46	0.20	1.15	0.19	NS
Water intake (ml/mouse)	Light	0.61	0.10	0.82	0.22	NS
	Dark	2.83	0.32	3.65	0.69	NS
VO_2 (ml/hr)	Light	91.8	2.58	84.94	2.58	P=0.097
	Dark	104.83	1.54	98.74	1.54	P<0.05
RER (VCO_2 / VO_2)	Light	0.83	0.01	0.82	0.03	NS
	Dark	0.98	0.01	0.92	0.02	NS

490 Data are means ± SEM (n = 8). LF-CAS, 10%kJ fat and 35%kJ sucrose with 20%kJ casein; LF-
491 WPI, 10%kJ fat and 35%kJ sucrose with 20%kJ whey protein isolate (WPI). NS; not significant;
492 VO_2, oxygen consumption; $_{VCO2,}$ Carbon dioxide consumption.
493

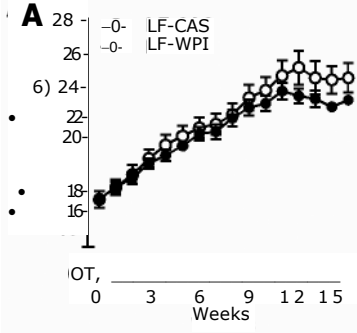
Table 3

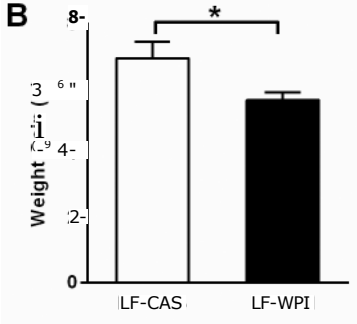
Intestinal gene expression in mice fed a LF-CAS or LF-WPI diet for 15 weeks.

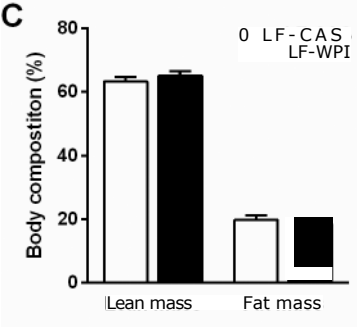
	LF-CAS	SEM	LF-WPI	SEM	*P* value
Duodenum					
IR	1.00	0.12	0.68	0.09	0.05
GLUT2	1.00	0.09	0.94	0.06	NS
IRS-1	1.00	0.09	0.84	0.07	NS
SGLT-1	1.00	0.07	0.83	0.05	0.07
CCK	1.00	0.05	0.76	0.15	NS
GIP	1.00	0.10	0.65	0.15	NS
FATP4	1.00	0.06	1.13	0.13	NS
Ileum					
IR	1.00	0.04	1.00	0.07	NS
GLUT2	1.00	0.21	1.89	0.31	<.05
IRS-1	1.00	0.06	1.13	0.17	NS
SGLT-1	1.00	0.09	0.99	0.10	NS
Proglucagon	1.00	0.07	1.13	0.17	NS
PYY	1.00	0.11	0.60	0.06	<.01
FATP4	1.00	0.08	0.77	0.04	<.05

Data are means ± SEM (n = 6-8). Values that differ significantly if $P < 0.05$; NS, non-significant. LF-CAS, 10%kJ fat and 35%kJ sucrose diet with 20%kJ casein; LF-WPI, 10%kJ fat and 35%kJ sucrose diet with 20%kJ whey protein isolate (WPI). Gene expressions are shown relative to the LF-CAS group which was set at 1.00. IR, Insulin receptor, GLUT2, Glucose transporter 2; IRS-1, Insulin receptor substrate 1; SGLT-1, Sodium-glucose co-transporter 1; CCK, Cholecystokinin; GIP, Glucose-dependent insulinotrophic peptide; PYY, Peptide YY; FATP4, Fatty acid transport protein 4.

Figure 1


A
28
26
6) 24
22
20
18
16
–0- LF-CAS
–0- LF-WPI
OT,
0 3 6 9 12 15
Weeks


B
8-
4-
2-
0
*
Weight
LF-CAS
LF-WPI


C
80
60
40
20
0
0 LF-CAS
LF-WPI
Body compostiton (%)
Lean mass
Fat mass

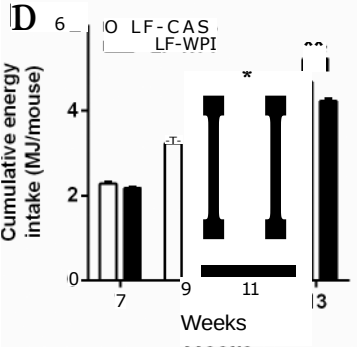
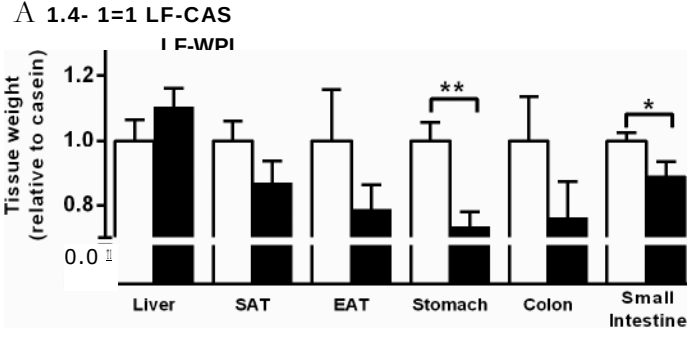

D
6
4
2
0
O LF-CAS
LF-WPI
*
Cumulative energy intake (MJ/mouse)
7
9
11
3
Weeks

figure 2


A 1.4- 1=1 LF-CAS
LF-WPI
Tissue weight
(relative to casein)
1.2
1.0
0.8
0.0
**
*
Liver
SAT
EAT
Stomach
Colon
Small
Intestine

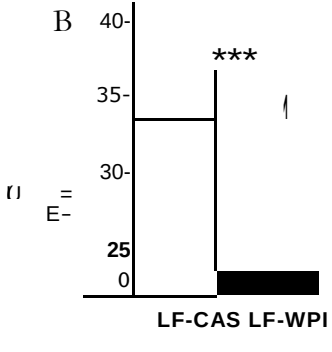

B
40-
35-
30-
25
0
***
LF-CAS LF-WPI

Figure 3

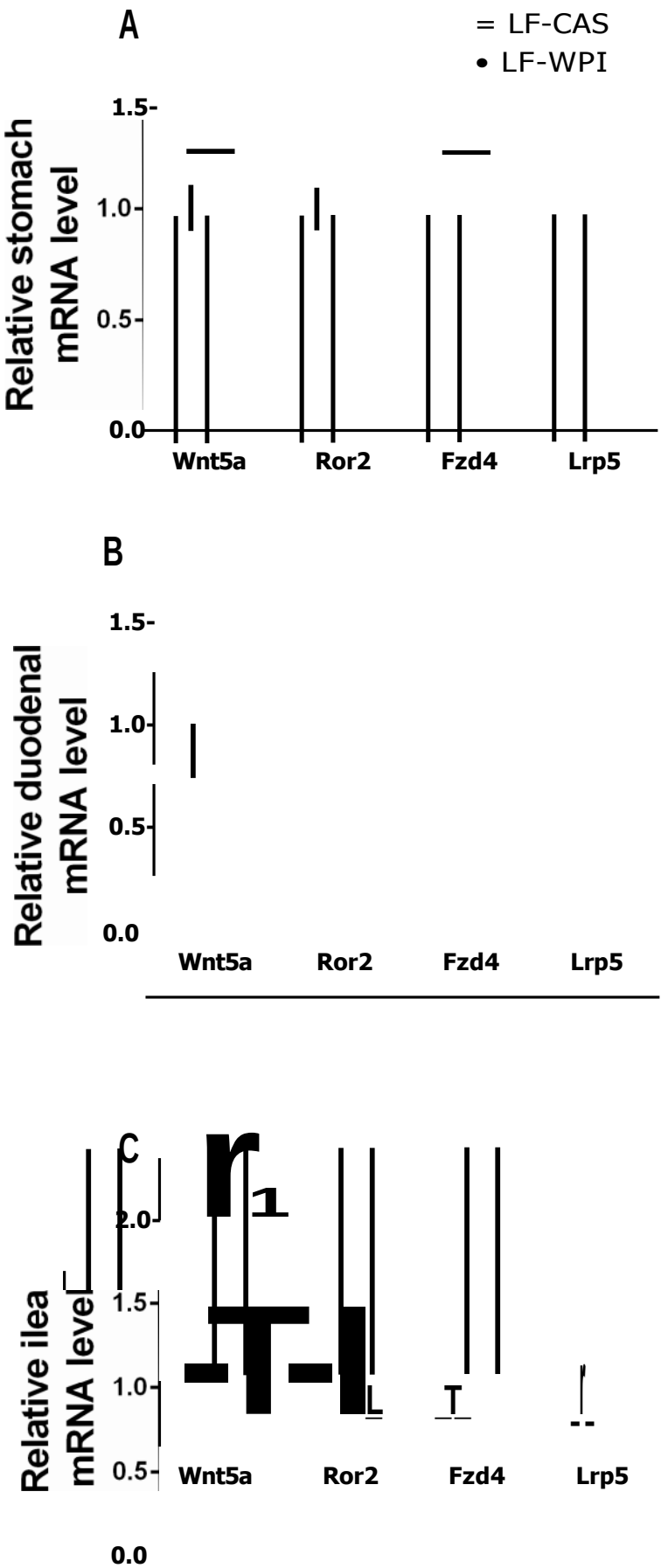
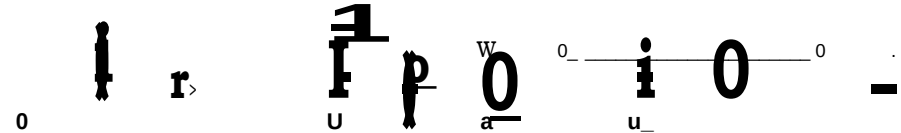

A
= LF-CAS
• LF-WPI
Relative stomach mRNA level
1.5
1.0
0.5
0.0
Wnt5a
Ror2
Fzd4
Lrp5
B
Relative duodenal mRNA level
1.5
1.0
0.5
0.0
Wnt5a
Ror2
Fzd4
Lrp5
C
Relative ilea mRNA level
2.0
1.5
1.0
0.5
0.0
Wnt5a
Ror2
Fzd4
Lrp5

Figure 4

w15



Supplementary table 1: Composition of the experimental diets

Contents (g)	LF-CAS	LF-WPI
Casein	200	0
WPI (>98% dry basis)	0	200
L-Cystine	3	3
Corn Starch	315	315
Maltodextrin 10	35	35
Sucrose	350	350
Cellulose, BW200	50	50
Soybean oil	25	25
Lard	20	20
Mineral mix S10026A	10	10
$CaHPO_4$	13	13
$CaCO_3$	5.5	5.5
$C_5H_6K_3O_7.1H_2O$	16.5	16.5
Vitamin mix V10001	10	10
Choline Bitartrate	2	2
Energy (kcal/g)	3.8	3.8
Protein (% kcal)	20	20
Carbohydrate (% kcal)	70	70
Fat(%kcal)	10	10

Diets formulated and produced by Research Diets Inc. (New Brunswick, NJ, USA)

LF, low fat diet; CAS, casein protein; WPI, whey protein isolate.

Supplementary table 2. Sequences of mouse specific primers used in real-time PCR analysis

	Forward primer (5 ’-3’)	Reverse primer (5 ’-3’)
Acetyl-CoA carboxylase (ACC)	5 ’-tgttgagacgctggtttgtagaa-3’	5 ’-ggtccttattattgtcccagacgta-3’
Beta-3 adrenergic receptor (β3-AR)	5 ’-cgccttcaacccggtcatctactg-3’	5 ’-ggtggactctgcctggcttcaac-3’
Cluster of differentiation 36 (CD36)	5 ’-tgatactatgcccgcctctcc-3’	5 ’-tttcccacactcctttctcctcta-3’
Carnitine palmitoyltransferase 1 a (CPT1 a)	5 ’-agacttccaacgcatgacagcactg-3’	5 ’-ctcggccccgcaggtagatg-3’
Carnitine palmitoyltransferase 1b (CPT1b)	5 ’-cgagaggggcggactgagactg-3’	5 ’-ggctaggcggtacatgttttggtg-3’
Cholecystokinin (CCK)	5 ’-gtgccgaggactacgaatacc-3’	5 ’-tctgggagtcactgaaggaaacac-3’
Fatty acid synthase (FASN)	5 ’-tccacctttaagttgccctg-3’	5 ’-tctgctctcgtcatgtcacc-3’
Fatty acid transport protein 1 (FATP1)	5 ’-ccggtgtggtggctgctcttctc-3’	5 ’-gctgccatctccccgccataaatg-3’
Fatty acid transport protein 4 (FATP4)	5 ’-tggcgtttcatccgggtcttcatc-3’	5 ’-gcaaacagcaggggcaccgtcttc-3’
Frizzled-4 (Fzd4)	5 ’-gctgactgtaggccgggaaagg-3’	5 ’-cggctgccaaaaaccaagtgag-3’
Glucose-dependent insulinotropic peptide (GIP)	5’-tctctgttgctggtgctcctgttc-3’	5 ’-gctctcctgtgcctctttgtcctc-3’
Glucose transporter 2 (GLUT2)	5 ’-tcctacttggcctatctgctgtgc-3’	5 ’-tgccctgacttcctcttccaac-3’
Glucose transporter 4 (GLUT4)	5’-ggcctgcccgaaagagtc-3’	5’-aggagctggagcaaggac-3’
Hormone sensitive lipase (HSL)	5’-ctattcagggacagaggcag-3’	5’-cgatgtggtcttttggggc-3’
Insulin receptor (IR)	5 ’-gatttccccaacgtgtcctctac-3’	5 ’-caatgcggtacccagtgaagtg-3’
Insulin receptor substrate 1 (IRS-1)	5’-gcgcaggcaccatctcaacaacc-3’	5’-gcacgcacccggaaggaacc-3’
Leptin	5 ’-ccccgcaccgctggaagtac-3’	5 ’-atgtgccctgaaatgcggtatg-3’
Lipoprotein lipase (LPL)	5 ’-tgctcccaacaatataagactcc-3’	5 ’-aaggccaggtgtttcaatc-3’
Lipoprotein-receptor related protein 5 (Lrp5)	5’ -cccgccctccaccttcttgctg-3’	5’-accgtcgtccttggccctcttgatg-3’
Peroxisome proliferator-activated receptor γ (PPARγ)	5’-tcaggtttgggcggatgc-3’	5’-tcagcgggaaggactttatgtatg-3’
Peptide YY	5 ’-ggacgcctaccctgccaaacca-3’	5 ’-agtgccctcttcttaaaccaaaca-3’
Pro-glucagon	5 ’-agggacctttaccagtgatgtga-3’	5 ’-acgagatgttgtgaagatggttgt-3’
Receptor tyrosine kinase-like orphan receptor (Ror2)	5’ -tggacggcaggtgaagtgg-3’	5’-gtctggccctgaacaatggtgata-3’
Sodium-glucose co-transporter 1 (SGLT-1)	5 ’-gagccccgcggttactgc-3’	5 ’-cctgcggctgctcctgtg-3’
Uncoupling protein 2 (UCP-2)	5 ’-ccatttcctgcaccccgatttacttcc-3’	5 ’-gctgggctggggatgaagatgaag-3’
Uncoupling protein 3 (UCP-3)	5 ’-acaggcccacacggtccagaacc-3’	5 ’-cccatcaggtcagtgcaaaacagagg-3’
Wnt5a	5 ’-cctgcctcgggactggttgtg-‘3	5 ’-cctacggcctgcttcattgttgtg-3’
β-actin	5 ’-agagggaaatcgtgcgtgac-3’	5 ’-caatagtgatgacctggccgt-3’
18-S	5 ’-aggaccgcggttctattttgttgg-3’	5 ’-atgctttcgctctggtccgtcttg-3’
YWHAZ	5 ’-cggagctgcgtgacatctgc-3’	5 ’-cctcggccaagtaacggtagtag-3’

Supplementary table 3: Skeletal muscle gene expression in mice fed a LF-CAS or LF-WPI diet for 15 weeks.

	LF-CAS	SEM	LF-WPI	SEM	*P* value
IR	1.00	0.19	1.01	0.08	NS
GLUT4	1.00	0.10	0.82	0.05	NS
IRS-1	1.00	0.08	0.81	0.04	0.06
CPT1b	1.00	0.13	0.85	0.06	NS
UCP-3	1.00	0.17	0.94	0.09	NS
LPL	1.00	0.14	0.87	0.16	NS
ACC	1.00	0.43	0.62	0.21	NS
CD36	1.00	0.14	1.05	0.15	NS

Data are means ± SEM (n = 8). Values differ significantly if $P < 0.05$; NS, non-significant. LF-CAS, 10% kJ fat, 35%kJ sucrose with 20%kJ casein protein; LF-WPI, 10% kJ fat, 35%kJ sucrose with 20%kJ whey protein isolate (20% kJ). Gene expressions are shown relative to the LF-CAS group which was set at 1.00. IR, Insulin receptor; GLUT4, Glucose transporter 4; IRS-1, Insulin receptor substrate 1; CPT1b, Carnitine palmitoyltransferase 1b; UCP-3, Uncoupling protein 3; LPL, Lipoprotein lipase; ACC, Acetyl-CoA carboxylase; CD36, Cluster of differentiation 36.

Supplementary table 4: Liver gene expression in mice fed a LF-CAS or LF-WPI diet for 15 weeks.

	LF-CAS	SEM	LF-WPI	SEM	*P* value
GLUT2	1.00	0.12	1.03	0.09	NS
IRS-1	1.00	0.12	0.93	0.06	NS
CPT1a	1.00	0.18	1.08	0.12	NS
UCP-2	1.00	0.07	1.07	0.08	NS
FASN	1.00	0.29	0.96	0.12	NS
ACC	1.00	0.14	1.10	0.14	NS
LPL	1.00	0.09	0.96	0.07	NS
CD36	1.00	0.20	0.92	0.07	NS

Data are means ± SEM (n = 7-8). Values that differ significantly if $P < 0.05$; NS, non-significant. LF-CAS, 10% kJ fat, 35%kJ sucrose with 20%kJ casein protein; LF-WPI, 10% kJ fat, 35%kJ sucrose with 20%kJ with whey protein isolate (20% kJ). Gene expressions are shown relative to the LF-CAS group which was set at 1.00. GLUT2, Glucose transporter 2; IRS-1, Insulin receptor substrate 1; CPT1a, Carnitine palmitoyltransferase 1a; UCP-2, Uncoupling protein 2; FASN, Fatty acid synthase; ACC, Acetyl-CoA carboxylase; LPL, Lipoprotein lipase; CD36, Cluster of differentiation 36