

RESEARCH ARTICLE | *Metabolic Control by Inflammation and Immunity*

Whey protein effects on energy balance link the intestinal mechanisms of energy absorption with adiposity and hypothalamic neuropeptide gene expression

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Nilaweera KN, Cabrera-Rubio R, Speakman JR, O'Connor PM, McAuliffe A, Guinane CM, Lawton EM, Crispie F, Aguilera M, Stanley M, Boscaini S, Joyce S, Melgar S, Cryan JF, Cotter PD. Whey protein effects on energy balance link the intestinal mechanisms of energy absorption with adiposity and hypothalamic neuropeptide gene expression. *Am J Physiol Endocrinol Metab* 313: E1–E11, 2017. First published March 21, 2017; doi:10.1152/ajpendo.00356.2016.—We tested the hypothesis that dietary whey protein isolate (WPI) affects the intestinal mechanisms related to energy absorption and that the resulting energy deficit is compensated by changes in energy balance to support growth. C57BL/6 mice were provided a diet enriched with WPI with varied sucrose content, and the impact on energy balance-related parameters was investigated. As part of a high-sucrose diet, WPI reduced the hypothalamic expression of pro-opiomelanocortin gene expression and increased energy intake. The energy expenditure was unaffected, but epididymal weight was reduced, indicating an energy loss. Notably, there was a reduction in the ileum gene expression for amino acid transporter SLC6a19, glucose transporter 2, and fatty acid transporter 4. The composition of the gut microbiota also changed, where *Firmicutes* were reduced. The above changes indicated reduced energy absorption through the intestine. We propose that this mobilized energy in the adipose tissue and caused hypothalamic changes that increased energy intake, acting to counteract the energy deficit arising in the intestine. Lowering the sucrose content in the WPI diet increased energy expenditure. This further reduced epididymal weight and plasma leptin, whereupon hypothalamic ghrelin gene expression and the intestinal weight were both increased. These data suggest that when the intestine-adipose-hypothalamic pathway is subjected to an additional energy loss (now in the adipose tissue), compensatory changes attempt to assimilate more energy. Notably, WPI and sucrose content interact to enable the component mechanisms of this pathway.

whey proteins; gut microbiota

MAMMALS ACHIEVE energy homeostasis by matching energy supply (from food consumed) with the summed energy demand

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of body tissues. This is achieved in part by sensing nutrient (energy) availability in the intestinal lumen, where their transport through a carrier-mediated mechanism in the circulatory system causes subsequent changes in the hypothalamic- and adipose-related mechanisms regulating energy balance (16, 35, 41). A growing body of evidence suggests that the gut microbiota also play important roles in energy balance regulation by influencing the related intestinal and hypothalamic cellular activities (10, 32) and by harvesting energy from ingested food and then providing it for host metabolism, including storage in the adipose tissue (3). Thus, in theory, manipulation of the gut microbiota, along with the intestinal nutrient-sensing and transport-related genes, has the potential to change the intestinal, hypothalamic, and adipose control of energy balance and body weight dynamics.

Whey proteins, including bovine serum albumin (BSA) and lactoferrin, are constituents of milk (19), with the bovine form having been part of the human diet since at least the Bronze Age (51). Because these proteins are now increasingly being used in infant formula, and in food products to improve muscle mass in athletes and the elderly (18), there is a need to understand the impact of their long-term use on energy balance. Indeed, whey protein isolate (WPI) has been shown to acutely (up to 3 h) lower energy intake in humans, possibly via the production of intestinal-derived satiety or satiation-related hormones (14), albeit the results are inconsistent (1). However, the long-term effects of WPI on energy balance do not appear to involve the production of these hormones (52), but rather can be attributed to a reduction in growth-related parameters, with WPI-fed mice presenting a reduced body length, which correlated with the plasma levels of insulin-like growth factor-I (47). We extended these findings by showing that individually housed mice fed for 15 wk a diet enriched with 20% WPI, 35% sucrose (high), and 10% fat (low) (all % by energy) had reduced small intestinal weight and length compared with controls fed a similar diet where casein (CAS) replaced WPI (26). This was accompanied by a reduction in cumulative energy intake and 24-h energy expenditure. The decreased

cumulative energy intake was reflected in the ileal expression of fatty acid transporter 4 (FATP4) and without a significantly altered body weight trajectory or changes in plasma levels of metabolites (glucose and triacylglycerols) and hormones (leptin and insulin) (26). These data led to the hypothesis that WPI affects the nutrient transport-related mechanisms in the intestine, thus reducing energy absorption, with subsequent changes in energy intake and expenditure acting to maintain the growth in the animals.

Because our previous study was conducted in individually housed mice for a prolonged period of time (26), and given the effects of this manipulation on metabolism, including the gut microbiota (34, 49), herein we used group-housed mice to test the above hypothesis. Because WPI has already been shown to cause an energy loss in the intestine by reducing fat absorption in humans (44), and in mice following 20 wk of diet intake (39), we envisaged that 1) this effect will arise because of changes in the gut microbiota involved in energy harvest and the intestinal transport-related genes (beyond what was shown previously), 2) the presumed energy loss will be reflected in physiological and cellular markers of fat catabolism (respiratory change ratio, adipose tissue weights, and related gene expression), caused by the depletion of specific nutrients in the plasma, and 3) that there will be parallel changes in hypothalamic neuropeptide gene expression to maximize the balance between energy intake and expenditure to support the growth in the animals. To this end, we investigated the time course of changes in the above parameters in relation to energy balance following WPI intake. To further interrogate the functionality of the underlying mechanisms, we also assessed the impact of sucrose content given that the protein and carbohydrate ratio influenced the gut microbiota and energy balance (25), where sucrose was shown to have a greater influence on energy intake and fat deposition in different adipose tissues compared with the intake of fructose or glucose (17).

MATERIALS AND METHODS

Experimental strategy. All procedures involving animals were approved by the University College Cork Animal Experimentation Ethics Committee (2011/005 and 2015/007) and were licensed under the Cruelty to Animals Act 1876 and the European Union (Protection of Animals Used for Scientific Purposes) Regulation 2012. Eighty-eight C57BL/6J male mice, age 3 wk old (Harlan), were group housed ($n = 4/\text{cage}$) on a 12:12-h light-dark cycle with ad libitum access to food and water throughout the study unless otherwise stated. During the initial 2-wk acclimatization period, mice were provided with a diet containing 10% fat, 35% sucrose, and 20% CAS (HS-CAS; no. D12450, all %values by energy; Research Diets) (Table 1). Subsequently, weight-matched mice received diets varying only in protein quality or sucrose content (Research Diets): 35% (high; HS) or 7% (low; LS) sucrose with either 20% CAS or WPI (Alacene 895; NZMP), where all groups were assigned six home cages ($n = 4/\text{cage}$) with the exception of the LS-CAS group, which had four home cages ($n = 4/\text{cage}$). The study lasted for 17 wk, with samples or analyses collected/performed in the light phase during the course of the study (study 1; Fig. 1A). In this study, at the 5- or 17-wk time points, mice were fasted for 8 h commencing at 2200 in the dark phase and then anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine), and blood samples were collected. Mice were euthanized by cervical dislocation, tissues were collected, and samples were stored at -80°C for subsequent analysis (Fig. 1A). In a separate study (study 2), aimed to measure energy loss in feces, group-housed mice were provided a HS-CAS ($n = 8$) or HS-WPI ($n = 16$) diet for 5 wk ($n = 4/\text{cage}$). The

Table 1. Diet composition

	Diets			
	HS-CAS	LS-CAS	HS-WPI	LS-WPI
Contents, g				
Casein	200	200	0	0
Whey protein isolate	0	0	200	200
L-Cysteine	3	3	3	3
Corn starch	315	506.2	315	506.2
Maltodextrin 10	35	125	35	125
Sucrose	350	68.8	350	68.8
Cellulose, BW200	50	50	50	50
Soybean oil	25	25	25	25
Lard	20	20	20	20
Mineral mix S10026A	10	10	10	10
Ca ₂ HPO ₄	13	13	13	13
CaCO ₃	5.5	5.5	5.5	5.5
C ₆ H ₅ K ₃ O ₇ ·1 H ₂ O	16.5	16.5	16.5	16.5
Vitamin mix V10001	10	10	10	10
Choline bitartrate	2	2	2	2
Composition				
Energy, kJ/g	15.9	15.9	15.9	15.9
Protein, %energy	20	20	20	20
Carbohydrate (%energy of which sucrose)	70 (35)	70 (7)	70 (35)	70 (7)
Fat, %energy	10	10	10	10

Diets were formulated and produced by Research Diets (New Brunswick, NJ). CAS, casein; HS, high sucrose; LS, low sucrose; WPI, whey protein isolate.

fecal samples were collected from individual mice during the light phase in weeks 3 and 4, and these were stored at -80°C for subsequent analysis.

Metabolic parameters. Body weight was measured weekly (Fig. 1A). Food intake was measured in group-housed mice until week 13 and converted to energy intake (using the energy density 15.9 kJ/g of the rodent diets; Research Diets). During weeks 13–15, mice from each group ($n = 8$; corresponding to two home cages) were randomly selected and placed individually in a TSE Phenomaster 8-Cage unit for 3 days. In each week, different groups were selected, where all the mice in each selected home cage were housed in the Phenomaster cages to collect the data. The data corresponding to food and water intake, substrate metabolism [represented by respiratory exchange ratio (RER)], oxygen consumption ($\dot{V}\text{O}_2$), and locomotor activity (in the x -, y -, and z -axis), were recorded in the final 24 h of the housing period, as detailed previously (24), with the exception that measurements were taken during a 1.5-min time period/cage every 13 min rather than 1 min/cage every 9 min as used previously.

16S rRNA gene sequence analysis. Fecal samples collected at different times were homogenized and processed using mechanical and chemical lysis. The 16S rRNA gene (V3–V4 region) was PCR amplified, and resulting amplicons were sequenced on the Illumina MiSeq platform using v3 sequencing chemistry with 2×250 -bp paired-end reads. Sequences were further filtered on the basis of quality (removal of low-quality nucleotides at the 3'-end) and length (removal of sequences with <200 nucleotides) with prinseq (42) and joined using fastq-join (<https://code.google.com/archive/p/ea-utils/>). The sequences were clustered with 97% identity level (calculated at the operational taxonomic unit) using the closed-reference *usearch* v7.0 algorithm (9) against the Greengenes database v13.8 (27). α - and β -Diversity was determined using QIIME (4).

Gene expression. Total RNA was extracted from hypothalamic blocks, epididymal adipose tissue (eWAT), and from 1-cm regions of the distal small intestine corresponding to the ileum (week 5 or 7) using the RNeasy Minikit and treated with DNase (Qiagen). cDNA was synthesized from 600 ng total RNA using the Superscript II Reverse Transcriptase kit (Life Technologies) and subjected to Real-

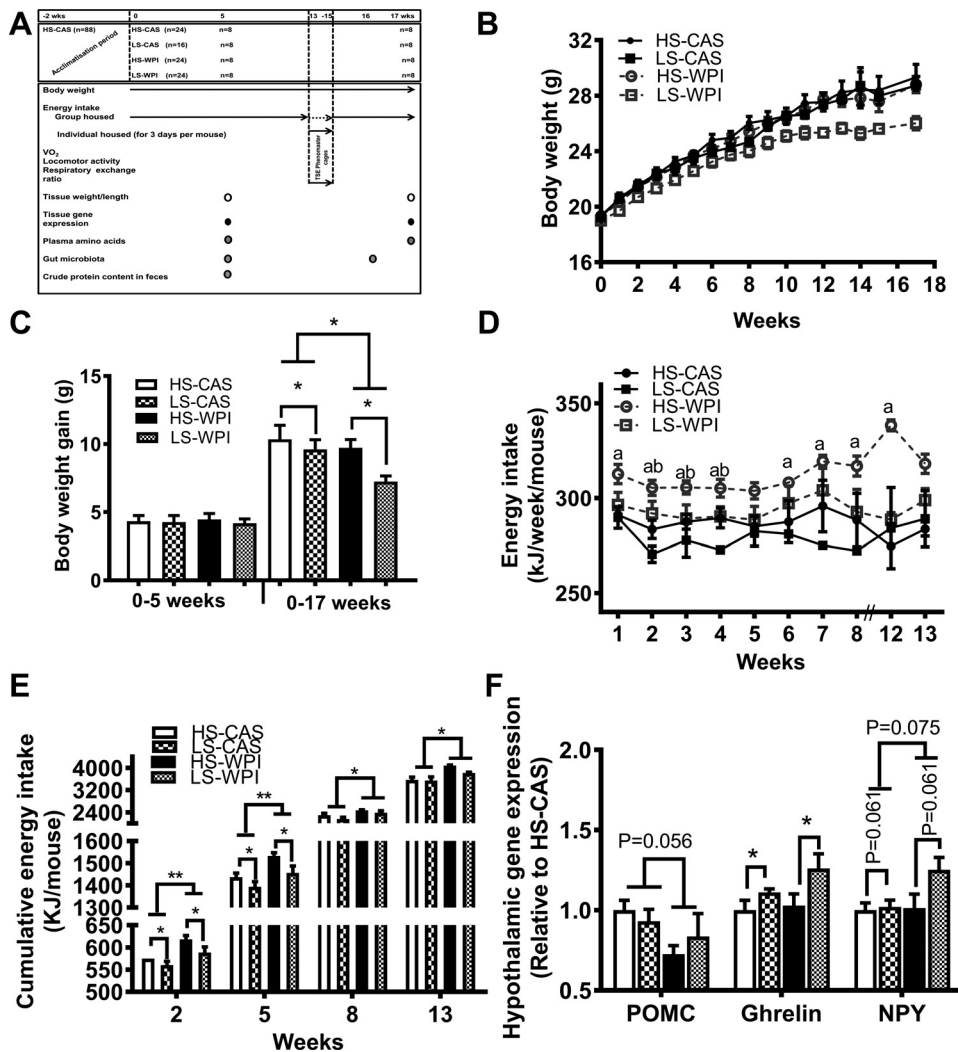


Fig. 1. Whey proteins affect energy intake and body weight gain. The experimental design in *A* was used to assess the impact of whey protein isolate (WPI) relative to casein (CAS) in high (HS) or low (LS) sucrose-enriched diets on body weight trajectory (*B*) and gain (*C*) in group-housed C57BL/6J mice. *D* and *E*: time course of changes in energy intake. *F*: hypothalamic expression of neuropeptide genes. * $P < 0.05$ or ** $P < 0.005$ by 2-way ANOVA, significant differences between WPI and CAS (*a*) and HS and LS (*b*). Data from $n = 24$ mice/group with the exception of LS-CAS, where $n = 16$. NPY, neuropeptide Y; *ob*, leptin gene; *Ob-r*, leptin receptor; POMC, pro-opiomelanocortin.

Time PCR (Roche) using SYBR Green Select Master Mix (Roche) as detailed previously (26). PCR primers and probes for cell cycle-associated cyclin D1 (*cD1*) and Kruppel-like factor 4 (*Klf4*) were designed using the Universal ProbeLibrary Assay Design Center (<https://www.roche-applied-science.com/sis/rtPCR/upl/adc.jsp>; Roche Applied Science, Indianapolis, IN). The authenticity of the PCR products was confirmed by DNA sequencing. Gene expression was calculated using $2^{-\Delta\Delta C_p}$ and normalized against the reference gene β -actin. The sequences of the primers are detailed elsewhere (24, 26, 29, 33).

Plasma levels of amino acids and leptin, and fecal total energy content. Plasma amino acid concentration was determined using the Jeol JLC-500/V amino acid analyzer (Jeol, Garden City, UK) fitted with a Jeol Na^+ high-performance cation exchange column, as detailed elsewhere (25, 28). Plasma leptin level was determined using the Mouse Leptin ELISA kit (Crystal Chem). To determine the total energy loss in feces, the samples collected from *study 2* were dried, weighed, pelleted using a Parr 2812 Pellet Press (Parr Instrument, Moline, IL), and analyzed using an Isoperibol Calorimeter 6200 instrument with a 1109A semimicro oxygen combustion vessel (Parr Instrument).

Statistical analysis. Data were analyzed by two-way ANOVA. \dot{V}_{O_2} data were analyzed by ANCOVA with body weight as a covariant as detailed previously (48). Statistical significance was established at $P < 0.05$. Data are presented as means with SE. For gut microbiota data, correspondence analysis (CA) was carried out at the family

level. Statistical differences between multiple samples were estimated by Kruskal-Wallis ANOVA and the multiple-test correction of Benjamini-Hochberg, and differences between two samples were estimated by Mann-Whitney test analysis and correction of Family-wise error rate, in both cases with the *R* statistical package (<https://www.r-project.org/>).

RESULTS

WPI increased energy intake but reduced body weight gain. The body weight gain over the first 5 wk was not affected by the diet (Fig. 1, *B* and *C*). In contrast, WPI and low sucrose content in the diet reduced body weight gain by *week 17* (WPI effect, $P = 0.048$ and sucrose effect, $P = 0.033$; Fig. 1, *B* and *C*). The WPI-fed mice, irrespective of the sucrose content, increased energy intake compared with CAS-fed controls (Fig. 1*D*), which was reflected cumulatively (*week 2*, $P = 0.002$; *week 5*, $P = 0.004$; *week 8*, $P = 0.017$; *week 13*, $P = 0.01$; Fig. 1*E*). Whereas lowering the sucrose content in WPI and CAS diets decreased energy intake in the initial 5 wk (*week 2*, $P = 0.035$; *week 5*, $P = 0.022$; Fig. 1, *D* and *E*), post-5 wk this effect was lost (Fig. 1, *D* and *E*). By housing mice individually in TSE Phenomaster cages during *weeks 13–15*, we determined that the higher energy intake observed in WPI-fed mice oc-

occurred in the light phase (12.6 ± 1.8 kJ in HS-WPI and 14.4 ± 1.1 kJ in LS-WPI vs. 10.1 ± 1.5 kJ in HS-CAS and 9.8 ± 1.7 kJ in LS-CAS; WPI effect, $P = 0.03$). The energy intake was reflected in the hypothalamic neuropeptide gene expression. Notably, WPI showed a trend toward a decrease in the expression of the anorexigenic neuropeptide pro-opiomelanocortin ($P = 0.056$) while similarly increasing the orexigenic neuropeptide Y ($P = 0.075$) relative to the CAS groups (Fig. 1F).

WPI reduced RER and adipose tissue weight. Measurement of substrate metabolism during weeks 13–15 revealed that the WPI group had a lower RER early during the light phase (0600 to 1000), seen initially between HS-WPI and HS-CAS and between HS-WPI and LS-WPI, and later between WPI and CAS groups irrespective of sucrose content (Fig. 2A). This indicated that the WPI group had a higher fat catabolism. In fact, by the end of the study period (week 17), WPI reduced both the interscapular brown adipose (iBAT) and eWAT weights compared with CAS-fed controls ($P = 0.042$ for iBAT and $P = 0.028$ for eWAT; Fig. 2B). Notably, the change in body weight rather than impact of diet was related to the altered adipose tissue weight ($P < 0.0001$ for both eWAT and iBAT). The reduction in eWAT weight in response to WPI intake was reflected in the expression of the macrophage marker cluster of differentiation 68 ($P = 0.024$; Fig. 2C). In contrast, the gene expression of fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC), both involved in lipogenesis, was increased in response to WPI intake ($P = 0.048$ for FASN and $P = 0.021$ for ACC; Fig. 2C). There were no significant effects of the diet on the eWAT expression of genes involved in fat catabolism, namely, uncoupling proteins 2 and 3, carnitine palmitoyltransferase Ia and b, peroxisome proliferator-activated receptor- α , hormone-sensitive lipase, and β -adrenergic receptor (data not shown).

WPI reduced ileal gene expression of nutrient transporters. To understand how the mismatch between energy intake and

body weight gain/fat mass in WPI-fed mice arises, we investigated the ileal gene expression for nutrient transporters. Notably, at week 5, WPI increased the expression of glucose transporter (GLUT) 2 ($P = 0.0009$; Fig. 3A). Additionally, the LS diet increased the expression of FATP4 ($P = 0.0007$), and the L-type amino acid transporter 4 (LAT4) ($P = 0.005$), with sodium-glucose transporter (SGLT1) showing a trend toward an increase at the same time point ($P = 0.085$; Fig. 3A). Although the methionine (Met) transporter SLC6a19 responded to LS in the CAS diet ($P = 0.002$; Fig. 3A), no such increase was observed in the LS-WPI, which was significantly lower compared with LS-CAS ($P = 0.037$; Fig. 3A). Prolonged WPI intake (up to 17 wk) decreased the expression of FATP4 ($P = 0.0008$) and SLC6a19 ($P = 0.047$), with 5-wk-responsive GLUT2 reaching baseline levels (Fig. 3B). In contrast to nutrient transporters, peptide YY (PYY) gene expression was not altered by diet at either time point (data not shown). Given the WPI effect on nutrient transporters, we assessed the total energy lost in fecal samples collected (in weeks 3–4) from mice fed HS-CAS or HS-WPI for 5 wk (study 2). This analysis revealed that the HS- and WPI-fed mice had a lower fecal energy content than HS- and CAS-fed controls (10.23 ± 0.34 kJ/g in HS-WPI vs. 12.37 ± 0.614 kJ/g in HS-CAS; $P = 0.011$). However, plasma Met concentration was reduced in HS-WPI compared with HS-CAS at week 5 (29.6 ± 1.14 nmol/ml in HS-WPI vs. 38.3 ± 1.07 nmol/ml in HS-CAS; $P = 0.002$; $n = 4$). At week 17, WPI maintained the effect on Met (25.3 ± 1.4 nmol/ml in HS-WPI and 24.7 ± 1.7 nmol/ml in LS-WPI vs. 27.5 ± 2.4 nmol/ml in HS-CAS and 30.7 ± 1.3 nmol/ml in LS-CAS; protein effect, $P = 0.031$) but, in addition, reduced threonine ($P = 0.041$; data not shown) and tyrosine ($P = 0.004$; data not shown), whereas both arginine and alanine ($P = 0.02$) increased in response to LS irrespective of the protein source (data not shown).

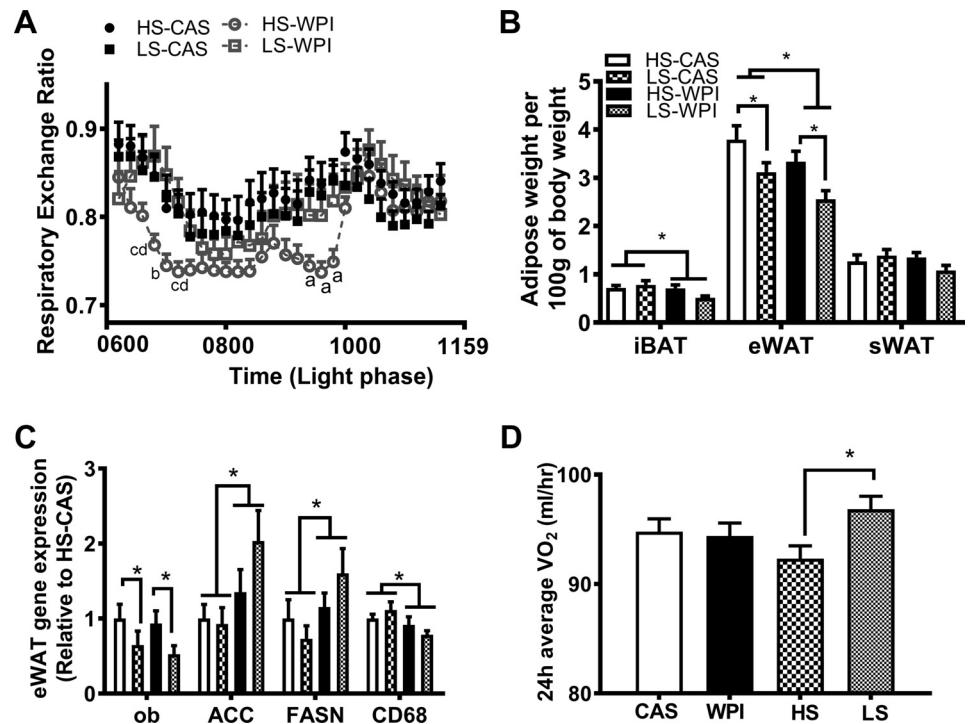


Fig. 2. Whely proteins reduce adiposity. Data were generated from individually housed C57BL/6J mice in TSE Phenomaster Cages at weeks 13–15, corresponding to respiratory exchange ratio (A) and energy expenditure (D) as measured by oxygen consumption ($\dot{V}O_2$). Also shown are the impact of 17 wk of diet intake on adipose tissue weight (B) and associated gene expression (C). * $P < 0.05$, significant differences between WPI and CAS (a), HS and LS (b), HS and LS-WPI (c), and HS-WPI and HS-CAS (d) (2-way ANOVA). ACC, acetyl-CoA carboxylase; CD68, cluster of differentiation 68; eWAT, epididymal adipose; FASN, fatty acid synthase; iBAT, interscapular brown adipose; sWAT, subcutaneous adipose.

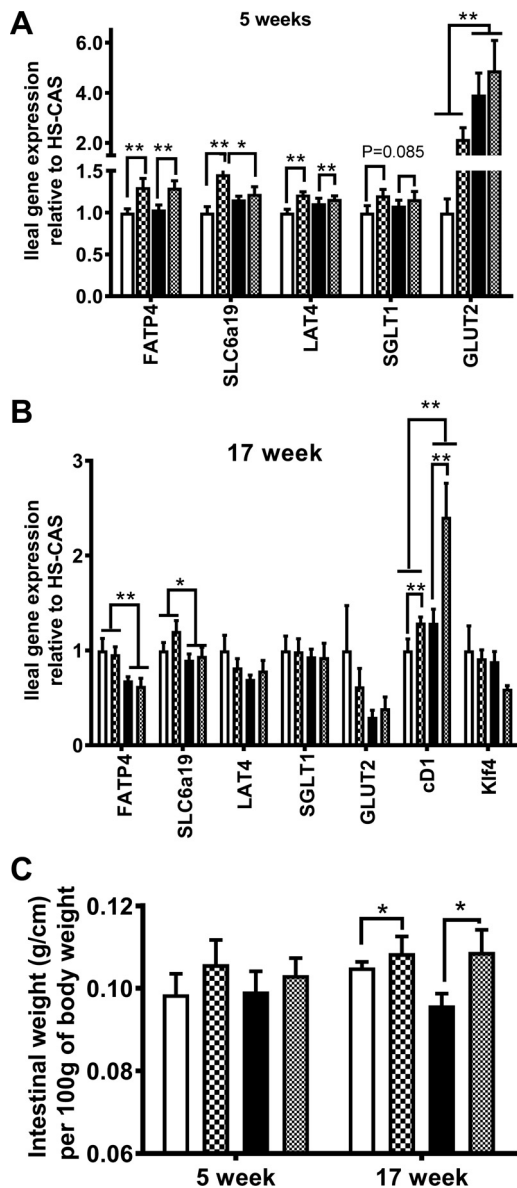


Fig. 3. Whey proteins affect expression of nutrient transport-related genes in the ileum. Data show the effect of diet on ileal gene expression measured at *week 5* (A) or *week 17* (B) in C57BL/6J mice. Also shown is the effect of diet on intestinal growth (C). * $P < 0.05$ and ** $P < 0.005$ by 2-way ANOVA; $n = 8$. cD1, cyclin D1; FATP4, fatty acid transporter 4; GLUT2, glucose transporter 2; Klf4, Kruppel-like factor 4; LAT4, L-type amino acid transporter 4; SLCa19, methionine transporter; SGLT1, sodium-glucose transporter 1.

Microbiota of WPI-fed animals was more stable over time and less influenced by sucrose than CAS-fed animals. Compositional analysis was carried out to determine the microbiota composition of stool samples collected at 5 and 16 wk, yielding an average of $128,178 \pm 75,076$ (SD) 16S rRNA high-quality sequences after quality filtering and trimming. Samples at each time point were grouped by sample type (i.e., HS-CAS, LS-CAS, HS-WPI, and LS-WPI). An examination of α -diversity across groups revealed significantly greater diversity in the HS-CAS diet group at *week 16* ($P < 0.005$, 2-way ANOVA) (Fig. 4A). Principal coordinates analysis of all reads at 97% similarity (Fig. 4, B and C) revealed that the microbiota of the WPI-fed and CAS-fed mice were distinct. This was apparent at

both time points but was more notable at *week 16* (Fig. 4C). In addition, although the microbiota of CAS-fed animals further separated according to the sucrose content of their diets, this alteration did not occur in samples collected from WPI-fed animals (Fig. 4C). The data also revealed taxonomic differences across the sample types. At the phylum level, it was apparent that *Bacteroidetes* are present in lower proportions and *Firmicutes* and *Actinobacteria* are present at higher proportions in HS-CAS relative to the other diet groups at *week 5* (Fig. 4D). At the family level, the HS-CAS group differed notably from other groups, containing greater proportions of *Streptococcaceae* and reduced proportions of *Rickenellaceae*, *Unclassified_Rickenellaceae*, and *Sutterellaceae* (Fig. 5A). Among the same samples, *Porphyromonadaceae* and *Unclassified_Sutterellaceae* were present in lower proportions in samples from CAS-fed, irrespective of the sucrose content, relative to WPI-fed animals (Fig. 5A). By *week 16*, *Tenericutes* were present in greater proportions in WPI-fed relative to CAS-fed animals (Fig. 4E). At this time, *Firmicutes* were, again, significantly overrepresented in HS-CAS groups relative to the other diet groups (Fig. 4E). At the family level, five families (*Unclassified_Sutterellaceae*, *Sutterellaceae*, *Anaeroplasmaceae*, *Unclassified_Porphyromonadaceae*, and *Porphyromonadaceae*) were present at significantly lower proportions in CAS-fed relative to WPI-fed mice (Fig. 5B). At the same time, *Streptococcaceae* and *Enterobacteriaceae* were found at significantly greater proportions in CAS-fed than in WPI-fed mice (Fig. 5B).

Sucrose content and WPI modulate adiposity, hypothalamic gene expression, and intestinal growth. Lowering the sucrose content in the CAS and WPI diets reduced eWAT weight at *week 17* ($P = 0.003$; Fig. 2B), which was reflected in the leptin (*ob*) gene expression ($P = 0.031$; Fig. 2C) and plasma levels of leptin (15.15 ± 1.39 ng/ml in HS-CAS, 10.03 ± 2.06 ng/ml in LS-CAS, 11.17 ± 1.82 ng/ml in HS-WPI, and 4.49 ± 0.61 ng/ml in LS-WPI; sucrose effect, $P = 0.0009$). Additionally, protein source also reduced plasma leptin level (WPI effect, $P = 0.0057$). The reduction in eWAT weight in response to low sucrose intake could be related to the higher energy expenditure, as measured by $\dot{V}O_2$ intake (Fig. 2D). In the hypothalamus, the lower sucrose content increased the gene expression of orexigenic ghrelin ($P = 0.019$; Fig. 1F), with neuropeptide Y showing a trend toward an increase ($P = 0.061$; Fig. 1F). Additionally, the intestinal weight per centimeter of the tissue expressed relative to body weight was increased in the LS compared with HS groups by *week 17* ($P = 0.036$; Fig. 3C). The intestinal length was unaffected in all groups (data not shown). The intestinal growth was reflected in the ileal gene expression for cell cycle-associated cD1 ($P = 0.002$; Fig. 3B). Additionally, WPI accentuated the (low) sucrose effect on cD1 gene expression ($P = 0.002$; Fig. 3B), where WPI and LS appeared to interact to enable greater expression of the gene ($P = 0.059$). Correspondingly, the gene expression of the inhibitor Klf4, which functions upstream of cD1, was reduced in the LS-WPI, albeit this did not reach significance (Fig. 3B). These data suggesting a link between eWAT, hypothalamic gene expression, and intestinal growth, which can be modulated by sucrose content and WPI, are further strengthened by our previous finding that obese mice fed BSA, a constitute protein in WPI, presented reduced adiposity, and increased cumulative energy intake compared

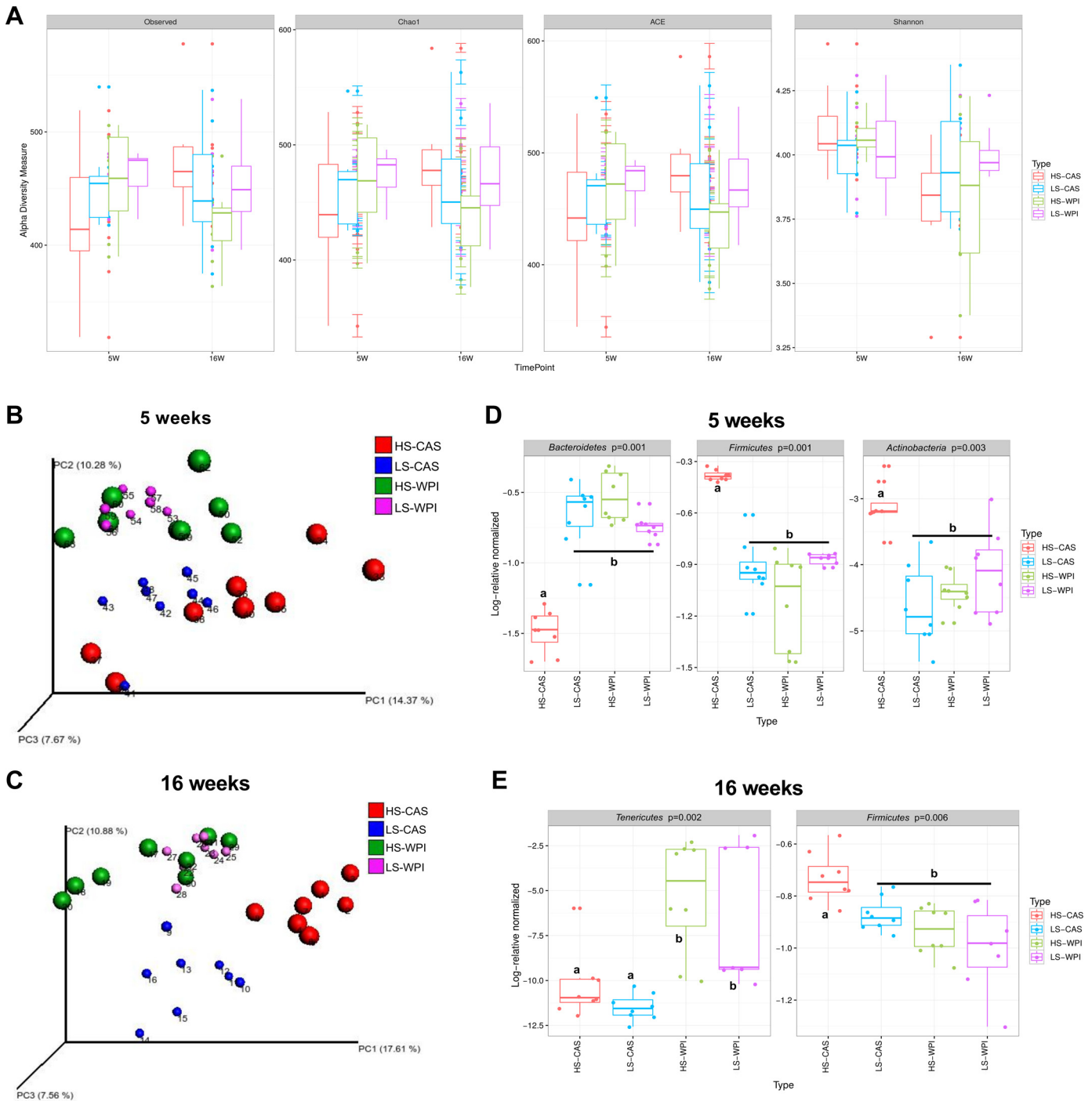


Fig. 4. Assessment of differences in the diversity or phylum-level taxonomy of the gut microbiota of animals in receipt of different diets. *A*: comparison of α -diversity between groups of mice fed HS-CAS (red), LS-CAS (blue), HS-WPI (green), and LS-WPI (purple). *B* and *C*: principal coordinate analysis (PCoA) of β -diversity at *week 5* (*B*) and at *week 16* (*C*). *D* and *E*: Kruskal-Wallis analysis of differences at phylum level at *week 5* (*D*) and *week 16* (*E*). Letters *a* and *b* denote statistically different groups ($n = 8$) with $P < 0.05$.

with mice fed CAS (29). Notably, the intestinal weight (expressed relative to 100 g of body wt) increased in the BSA group compared with CAS group (3.06 ± 0.26 g in BSA vs. 2.35 ± 0.07 g in CAS; t -test with $n = 8$; $P = 0.021$).

DISCUSSION

A key finding of this study was that group-housed mice fed HS diet with WPI consistently consumed more energy

than CAS-fed controls. Because there were also no major changes in energy expenditure, and the body weight gain and adipose tissues were reduced, the data suggested that energy was being lost somewhere, most likely via malabsorption in the intestine and/or secretion in the urine. Lowering the sucrose content accentuated the energy loss, which further reduced the body weight gain and adipose tissue weight.

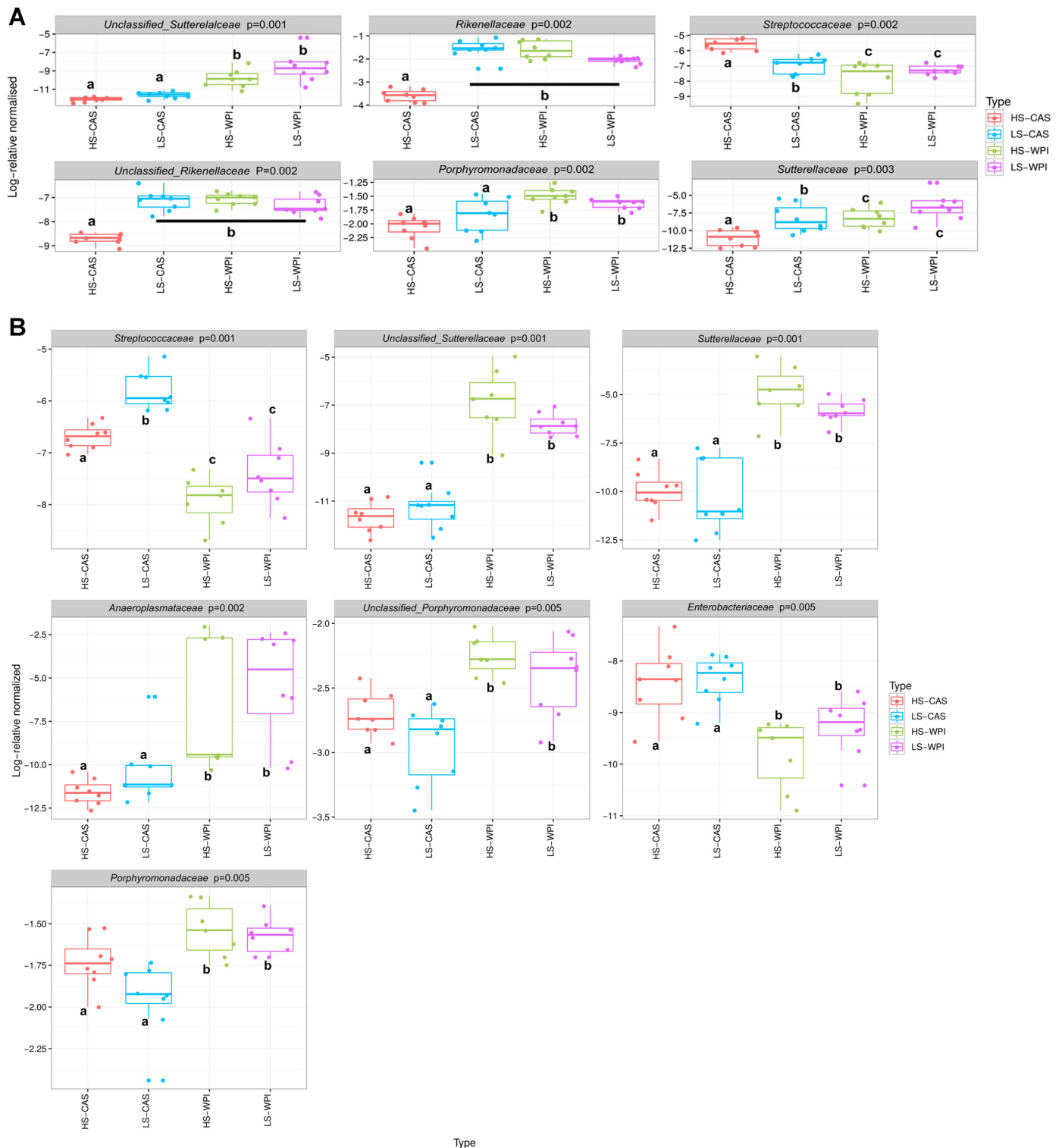


Fig. 5. Assessment of differences in the family level of the gut microbiota of animals in receipt of different diets. Kruskal-Wallis-based analysis of differences at the family level between HS-CAS (red)-, LS-CAS (blue)-, HS-WPI (green)-, and LS-WPI (purple)-fed animals at *week 5* (A) or *week 16*. (B). Letters *a* and *b* denote statistically different groups with $P < 0.05$.

Previous studies investigating the impact of whey proteins on energy balance have yielded inconsistent results with regard to impact on energy intake and body weight gain (37, 38, 52). The differences can be related to duration of feeding [28 days (37) vs. 9 wk (52)], the protein-derived energy content in the diets

[40% (37) vs. 55% (38)], and carbohydrate content in whey protein diets (25, 38). Although the current data are in agreement with the latter suggestion, specifically suggesting an interaction between WPI and sucrose, a clear discrepancy is the higher energy intake and reduced body weight gain seen here

compared with rat studies (38, 52) as well as our previous murine study, where WPI reduced cumulative energy intake and body weight gain (26). The discrepancy could be related to the method of housing, since the animals used in these previous studies were housed singly. Notably, in our previous study, the single-housed mice fed WPI had a reduced intestinal length and reduced ileal gene expression for PYY and FATP4 (26), whereas group-housed mice used in the current study only reduced FATP4 gene expression. Given the (contrasting) effects of PYY on anabolic and catabolic pathways (43), and the role played by FATP4 in intestinal lipid transport (2), the data suggest a potential interaction between the housing environment and whey proteins on intestinal morphology (length) and cellular activity linked to energy balance regulation. In fact, Tranberg et al. showed that group-housed mice fed WPI as part of a 60% energy high-fat diet (HFD) also increased energy intake and reduced body weight gain, similar to the current study, but, in this instance, the effect was transient, only seen in the first week of diet intake (47). This may be because of the HFD-induced changes in the intestinal cellular activity (7) impacting WPI effects mediated through this tissue. In the current study, using group-housed mice, we explored the effect of 10% energy fat with WPI on intestinal nutrient transporter-related genes and the gut microbiota involved in nutrient/energy absorption and assessed how any changes relate to energy balance.

Whey proteins affect intestinal mechanisms linked to energy absorption. Given that sucrose has been shown to increase energy intake compared with water intake (11), as well as fructose and glucose intake (17), we predicted that variation in the sucrose content would further alter nutrient transporter-related gene expression in response to the level of energy intake, and this, in turn, would provide a way to scrutinize the functionality of any WPI effects on these genes. It is noteworthy that the effects of sucrose on energy balance appear to depend on the form in which it is provided for consumption (solid vs. liquid) (17), duration of intake (45), and the stage of development (postweaning vs. adulthood) (11). In the current experimental design, LS- relative to HS-CAS intake increased the ileal gene expression for FATP4 and Met transporters expressed in the apical (SLC6a19) and basolateral (LAT4) sides (23) at 5 wk and caused a trend toward an increased expression of the glucose transporter (SGLT1) expressed in the apical side of the intestine. The gene expression changes appear to be part of a mechanism to compensate for the reduced energy intake in the LS- compared with HS-CAS groups because, once the energy intake was normalized after 5 wk, presumably caused by a postpubertal preference for carbohydrate intake (20) coupled with a higher preference for sucrose compared with glucose (17), so did the gene expression profile by *week 17*. This suggested that the above-mentioned genes encoding nutrient transporters were sensitive to ingested quantities of lipids (by FATP4) (2), Met (by SLC6a19 and LAT4) (23), and glucose (by SGLT1) (12) and that their gene expression can be modulated by varying the sucrose content in the diet. Changing the protein quality to whey did not alter the sucrose effect on energy intake and ileal gene expression at 5 wk. At this time point, the intestine responded to the WPI-induced increased energy intake by promoting greater energy absorption compared with CAS. This was supported by the following evidence: 1) mice fed HS-WPI diet had

reduced energy content in feces compared with the HS-CAS group, indicating a greater macronutrient absorption in the former group and 2) GLUT2 gene expression increased in WPI groups compared with CAS groups at 5 wk, in line with glucose passage across the intestinal cells providing the signal to induce GLUT2 gene transcription (12, 13). Based on the responsiveness of genes to the dietary macronutrient content at *week 5*, we suggest that the reduction in GLUT2 to baseline levels in both WPI groups by *week 17* is a reflection of reduced nutrient absorption at this time point. Similarly, the time course of reduction in FATP4 and SLC6a19 in WPI-fed mice suggested a reduced fat and Met absorption through the intestine, consistent with the metabolite data, albeit the possibility exists that the change in plasma Met reflects tissue utilization of this amino acid in the WPI compared with CAS group. Together, our data suggest that WPI reduces the responsiveness of ileal nutrient transporters to nutrient availability in the lumen over time despite consuming more energy, thus providing a potential mechanism for the energy loss in the intestine shown previously in mice (39) and humans (44). This potential mechanism may work in conjunction with the known energy loss in urine, specifically via excretion of tricarboxylate cycle intermediates (21).

Given the proposed role of the gut microbiota in energy harvest (3, 5), we previously used the HFD-fed mouse model to investigate the involvement of the gut microbiota in mediating WPI effects. This work showed that WPI (20% by energy) reduced HFD (45% energy)-induced weight gain and that increasing the protein content (up to 40% energy) in the diet led to a dramatic reduction in weight gain, achieving a similar body weight trajectory as low fat with CAS-fed controls (25). Notably, the composition of the gut microbiota in the 40% WPI group was distinct from that of the low-fat-fed controls (25). Using the same low-fat-fed mouse model with matching protein contents, we now show that the WPI-associated microbiota was distinct and more stable over time than CAS-fed controls, which, in turn, were even more considerably affected by the sucrose content in the diet. Interestingly, an in-depth analysis showed changes in the microbiota with links to energy balance. Notably, at the phylum level, a high ratio of *Firmicutes* to *Bacteroidetes* and increased levels of the *Actinobacteria* have been associated with positive energy balance (3, 5, 22, 50). In this regard, it is notable that, at *week 5*, the *Bacteroidetes* were elevated and *Firmicutes* and *Actinobacteria* were reduced in both WPI groups. The changes were reflected at the family in the proportions of *Rikenellaceae* (*Bacteroidetes*) and *Streptococcaceae* (*Firmicutes*) present and are consistent with previous findings associating reduced *Rikenellaceae* or elevated *Streptococcaceae* with the obese state (6, 15). The above changes in the phyla/families are consistent with the resistance shown by HS- and LS-WPI-fed mice to gain weight despite consuming more energy. Surprisingly, the *Sutterellaceae* family, which was previously shown to be associated with positive energy balance (15), was significantly increased by WPI intake. The growth of distinct microbial populations with potential opposing effects on energy balance is intriguing, and this effect is seen at the later time point, where WPI maintained the reduced levels of *Firmicutes*/*Streptococcaceae* but increased the proportion of *Tenericutes*/*Anaeroplasmataceae* known to be associ-

ated with positive energy balance (36). Given the effects of WPI to reduce nutrient absorption (39, 44) and simultaneously increase energy intake, shown here, presumably to counteract the intestinal energy loss and support growth, this raises the possibility of a dual mode of action for gut microbiota working either in combination with or independent of the nutrient transport-related genes.

Adipose tissue energy loss is linked to hypothalamic gene expression and intestinal growth. Irrespective of the protein source, lowering the sucrose content increased energy expenditure. In the CAS diet, this was associated with a reduced epididymal weight and plasma leptin. Concurrently, there was an increased hypothalamic orexigenic ghrelin gene expression and an increased intestinal growth (weight per centimeter expressed relative to body weight), which was reflected in the ileal expression of a marker of cell division (cD1). The data suggest a signaling mechanism linking adiposity, hypothalamic neuropeptide gene expression, and intestinal growth, where the latter two changes may act to increase energy assimilation to counteract the energy loss in the adipose tissue. In fact, energy restriction, lactation, and cold exposure, each of which reduces adiposity and plasma leptin, also increased energy intake and the intestinal weight (8, 30, 40, 46). By changing the protein to WPI, and bearing in mind the effect on the intestinal mechanisms of energy absorption and presumed energy loss, the above effects of the low sucrose were accentuated. We propose that BSA, a constituent protein within WPI, provides some of the bioactivity to WPI because our previous work showed that BSA reduced adiposity and plasma leptin and increased cumulative energy intake in obese mice (29), and the data presented here from that study extended the link to include the growth of the intestine. The signaling mechanism may involve leptin given that leptin-deficient (*ob/ob*) mice show increased intestinal weight (31).

In conclusion, we present mechanisms linking intestinal growth and energy absorption, with adiposity and the hypothalamic control of energy balance, where whey proteins and varying levels of sucrose activate components of these mechanisms.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.N.N. conceived and designed research; K.N.N., R.C.-R., J.R.S., P.M.O.C., A.M., C.M.G., E.L., F.C., M.A., M.S., S.B., S.J., S.M., J.F.C., and P.D.C. performed experiments; K.N.N., R.C.-R., J.R.S., P.M.O.C., A.M., C.M.G., E.L., F.C., M.A., M.S., S.B., S.J., S.M., J.F.C., and P.D.C. analyzed data; K.N.N., R.C.-R., J.R.S., P.M.O.C., A.M., C.M.G., E.L.,

F.C., M.A., M.S., S.B., S.J., S.M., J.F.C., and P.D.C. interpreted results of experiments; K.N.N., R.C.-R., M.A., M.S., S.B., S.J., S.M., and P.D.C. prepared figures; K.N.N., R.C.-R., J.R.S., and P.D.C. drafted manuscript; K.N.N., R.C.-R., J.R.S., P.M.O.C., A.M., C.M.G., E.L., F.C., M.A., M.S., S.B., S.J., S.M., J.F.C., and P.D.C. edited and revised manuscript; K.N.N., R.C.-R., J.R.S., P.M.O.C., A.M., C.M.G., E.L., F.C., M.A., M.S., S.B., S.J., S.M., J.F.C., and P.D.C.

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