


## ORIGINAL ARTICLE

# Establishing a model for childhood obesity in adolescent pigs

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Received 15 November 2017; revised 27 March 2018; accepted 1 April 2018

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**Summary****Objective**

Rising worldwide prevalence of obesity and metabolic diseases in children has accentuated the importance of developing prevention and management strategies. The objective of this study was to establish a model for childhood obesity using high-fat feeding of adolescent pigs, as pigs have a longer developmental period and are physiologically more similar to humans than rodents.

**Methods**

Crossbred pigs were fed a high-fat diet (HFD) or low-fat diet ( $n = 6/\text{treatment}$ ) from postnatal day 49 to 84. On postnatal day 84, an oral glucose tolerance test was performed, jugular blood sampled to determine lipopolysaccharide levels and plasma lipids, intestinal digesta collected to characterize microbial and metabolite composition and back fat and intestinal tissue assayed for gene expression.

**Results**

Five-week HFD increased weight gain and back fat thickness, caused dyslipidaemia and impaired glucose tolerance and increased expression of genes in back fat suggesting inflammation. HFD pigs had distinct proximal colon microbiota with 48% reduction ( $P < 0.05$ ) in Bacteroidetes and increased expression of pro-inflammatory genes interleukin-18 and tumour necrosis factor in ileum ( $P < 0.05$ ).

**Conclusions**

These findings indicate that adolescent pigs should be considered a suitable model for childhood obesity, because short-term HFD feeding is sufficient to induce obesity and glucose intolerance, recapitulating disease characteristics in adolescent pigs.

**Keywords:** Childhood obesity, dyslipidaemia, impaired glucose tolerance, microbiome.

## Introduction

Obesity is characterized by excess body fat and is often accompanied by inflammation and insulin resistance (1). Approximately one third of 5 to 17-year olds, an estimated 1.6 million, were classified as overweight (19.8%) or obese (11.7%) in 2009 to 2011 in Canada (2), and 17% of 2–19-years olds (~12.7 million) were classified as obese in the United States in 2011 to 2014 (3). Obesity in children poses a major health threat and may lead to the development of type 2 diabetes, cardiovascular diseases and cancer (4). In order to develop strategies for prevention and treatment of childhood obesity, it is

fundamental to understand the disease pathophysiology using an appropriate animal model.

Rodent models are commonly used in obesity research; however, vast differences in eating habits, metabolism and physiology between rodents and humans impede the translation of findings into prevention and treatment strategies for humans (5). In addition, rapid disease development in rodents is unsuitable to study obesity development in detail. Alternatively, pigs have a relatively long developmental period, are physiologically similar to humans, have comparable pancreas morphology and share similar gastrointestinal structure and function (6,7). Dietary manipulation is adequate to induce

obesity in adult swine models with a 6-month high-fat/high-sucrose diet inducing insulin resistance, mild diabetes and atherosclerotic lesions in minipigs (8). When fed high-fat diet (HFD) for 40 weeks, Ossabaw swine develop obesity, glucose intolerance and dyslipidaemia (9). On the other hand, increased propensity for obesity and metabolic disease is now associated with early life antibiotics (10). In a previous study, early life antibiotic exposure in piglets altered metabolic regulation with a standard diet, however failed to change fat deposition (11). It was expected that the lack of difference in adiposity was due to the absence of a dietary challenge. To date, no models of childhood obesity induced by short differential feeding periods have been established in pigs.

Persons with lean and obese phenotypes have major distinctions in their gut microbial profiles (12,13). Pigs, with similar gastrointestinal structure and function to humans, may recapitulate this feature. However, previous studies using HFD-treated pig models have failed to properly characterize gut microbial composition (8,9) or have characterized microbial composition without observing signature phenotypes of obesity (14,15). Therefore, it was hypothesized that a 5-week HFD is sufficient to induce obesity and alter gut microbial composition in adolescent pigs. The objectives of the current study were to develop an adolescent pig model for childhood obesity and evaluate disease characteristics of the resulting model.

## Methods

### Animals and experimental design

The animal study was approved by the Animal Care and Use committee of the University of Alberta according to guidelines of the Canadian Council on Animal Care and was conducted at the Swine Research and Technology Centre (Edmonton, AB, Canada).

A total of 12 crossbred pigs (Duroc × Large White/Landrace), reared together after weaning on postnatal day (PND) 21, were selected on PND 35 and moved to individual metabolic pens for a 2-week acclimation period with *ad libitum* access to a standard low-fat diet (LFD, Table S1) and water. On PND 49, an oral glucose tolerance test (OGTT) was conducted to ensure similar glucose tolerance of all pigs. Pigs were randomly assigned ( $n = 6/\text{treatment}$ ) to a HFD (fat: 21%, w/w) or LFD (fat: 4.3%, w/w) (Table S1) and were fed *ad libitum* for 5 weeks. Essential amino acids, vitamins and minerals in both diets were balanced based on metabolizable energy. Animals were housed at 22–25°C with a 12-h photoperiod. Body weights were recorded weekly from birth, and feed intake was recorded daily from PND 38 to 84.

### Blood glucose and plasma insulin measurement

On PND 77, OGTTs were performed after an overnight fast as previously described (11). Briefly, pigs were fed 50 g of LFD mixed with 2 g kg<sup>-1</sup> bodyweight glucose solution. Upon finishing the meal (time 0), blood glucose was measured from the ear vein in 15–30 min increments over 150 min. Additional blood samples were collected from –15 to 60 min in 15 min increments for plasma. Samples were centrifuged at 1,500 rpm for 10 min at 4°C and plasma collected and stored at –80°C until assayed for insulin by enzyme-linked immunosorbent assay, according to the manufacturer's instructions (ALPCO Diagnostics, Salem, N.H., USA). Blood glucose and plasma insulin concentrations were plotted as a function of time, and area under the curve (AUC) was calculated (11).

### Animal euthanasia and sampling

On PND 84, pigs underwent general anaesthesia with a mixture of ketamine-HCl (16 mg kg<sup>-1</sup>, Ketalean; Bimeda-MTC, Cambridge, ON, Canada), xylazine (2.2 mg kg<sup>-1</sup>, Rompun; Bayer Inc., Toronto, ON, Canada) and azaperone (6 mg kg<sup>-1</sup>, Stresnil, Janssen Pharmaceutica, Beerse, Belgium), followed by 5% isoflurane (Isoflo, Abbott Laboratories Ltd., Saint-Laurent, Quebec, Canada). Blood samples were collected aseptically to measure circulating lipopolysaccharide (LPS) concentrations. Subsequently, pigs were immediately exsanguinated. Digesta, mucosa and tissue samples of the ileum (25-cm proximal of the ileo-caecal junction) and proximal colon (15-cm distal of ileo-caecal junction) and back fat were collected, snap-frozen in liquid nitrogen and stored at –80°C. Back fat thickness between the dermis and muscle layers was measured at the fourth to last rib.

### Plasma lipids

Plasma triglyceride concentration was measured using Triglyceride-SL assay kit (Sekisui Diagnostics, Lexington, MA, USA) according to manufacturer's instructions. Total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol (LDL-c) were determined as per manufacturer's instructions (Wako Diagnostics, Richmond, Canada).

### Circulating lipopolysaccharide

Plasma LPS concentrations were measured using PYROGENT-5000 kit as per manufacturer's instructions (Lonza, Mississauga, Canada). Samples were diluted 1:80 and heated for 10 min at 70°C. The absorbance was measured at 340 nm min<sup>-1</sup> for 1 h at 37°C using a

SpectraMax® M3 Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

### Intestinal alkaline phosphatase activity

Alkaline phosphatase activity in the ileal mucosa was measured by SensoLyte® pNPP Alkaline Phosphatase Assay Kit according to manufacturer's instructions (AnaSpec, USA). Absorbance was measured at 405 nm using SpectraMax M3 Microplate Reader. Intestinal alkaline phosphatase (IAP) activity was normalized to protein concentrations of homogenized samples, measured using a Pierce BCA Protein Assay (Thermo Fisher Scientific).

### Short chain fatty acid concentrations

Proximal colon and ileal digesta were analysed for short chain fatty acid (SCFA) using gas chromatography. Briefly, digesta were mixed with 25% phosphoric acid at a ratio of 1:4 (w:v). Samples were vortexed and centrifuged at 3,500 rpm for 10 min at 4°C. Supernatant was collected and centrifuged at 15,000 rpm for 10 min at 4°C, then filtered through 0.45-µm filters (Thermo Fisher Scientific) and mixed with internal standard solution (24.5 mmol L<sup>-1</sup> isocaproic acid) at a ratio of 4:1. Samples were transferred into gas chromatography vials and injected by an autosampler (Model 8400; Varian Inc., Walnut Creek, CA) into a Stabilwax-DA column (Restek Corporation, Bellefonte, PA) on a Varian gas chromatograph (Model 3800; Varian Analytical Instruments, Palo Alto, CA). Peak integration was evaluated using Galaxie Software (Varian Inc.) and normalized by the weight of each sample used.

### RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted from the ileum and proximal colon using the GeneJET RNA Purification Kit (Thermo Fisher Scientific) as previously described (11). TRI Reagent® (Sigma, Oakville, ON, Canada) method was used to extract RNA from back fat (16). cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative real-time polymerase chain reaction (PCR) was performed as previously described (11) using primers listed in Table S2. Data were analysed using the comparative CT ( $2^{-\Delta\Delta CT}$ ) method as described previously using glyceraldehyde phosphate dehydrogenase as the housekeeping gene.

### DNA extraction and 16S rRNA sequencing

Total DNA from ileum and proximal colon digesta was extracted using QIAamp DNA Mini Stool Kit (Qiagen,

Inc.) following the manufacturer's instructions, with the addition of a bead-beating step (FastPrep instrument, MP Biomedicals, Solon, OH). DNA concentration was determined by Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific). DNA (2.5 µL, 5 ng µL<sup>-1</sup>) was amplified targeting V3–V4 regions of the bacterial 16S rRNA genes using the universal primers. PCR was performed using KAPA HiFidelity Hot Start Polymerase (Kapa Biosystems, Inc. Wilmington, MA, USA): 5 min at 95°C, 25 cycles of 20 s at 98°C, 15 s at 55°C, 1 min at 72°C, hold at 4°C. Subsequently, PCR products were purified using AMPure XP beads (Beckman Coulter, Inc. Mississauga, ON, Canada), then dual indices and Illumina sequencing adapters were attached using Nextera XT Index Kit (Illumina, Inc. Victoria, BC, Canada). Cycling conditions were 5 min at 95°C, 10 cycles of 20 s 98°C, 15 s 55°C, 1 min 72°C, hold at 4°C. PCR products were purified again, concentration measured and diluted to 4 nM. Aliquot of 5 µL of each diluted DNA was mixed for pooling libraries. Pooled library (5 µL) was size-selected and denatured with NaOH, diluted to 4 pM in Illumina HT1 buffer, spiked with 20 PhiX and heat denatured at 96°C for 2 min prior to loading. A MiSeq 600 cycle v3 kit was used to sequence each sample on an Illumina MiSeq sequencer. Nextera adapter sequences were used for run trimming.

### Sequence data processing

Sequence data were analysed using a QIIME pipeline (MacQIIME 1.8.0 OS10.10) (17). PANDAseq was used for quality filtering and to assemble paired end reads into contigs with miscalled or uncalled bases discarded (18). Resulting sequences were cleared of chimeras and singletons using UCHIME and UPARSE workflows, respectively, and were subsequently clustered into operational taxonomic units (OTUs) having >97% similarity with USEARCH (19). Taxonomy was assigned using QIIME default setting, Ribosomal Database Project classifier V2 (20). Alpha-diversity and beta-diversity estimations were conducted using the QIIME workflow `core_diversity_analysis.py` (21). Briefly, alpha diversity was estimated using Inverse Simpson and Shannon indices (22). Differences in microbial communities between groups were investigated using nonmetric multidimensional scaling phylogeny-based Bray–Curtis dissimilarity (Paleontologic Statistics Software Package, Ø. Hammer et al., 2016).

### Statistical analysis

Data were presented as means and standard deviation of the mean (stdev). Two-way ANOVA with repeated variable

of time was used to compare glucose, insulin, body weight, feed intake and energy intake between treatments followed by Bonferroni post hoc tests. Student's *t* test was used to compare glucose and insulin AUC, LPS concentrations, IAP activity, alpha diversity and gene expression between groups. Relative abundance of microbial taxonomy was non-normally distributed and thus analysed using Kruskal–Wallis rank sum test. Data were analysed using GraphPad Prism v. 6.02 (La Jolla, CA) and SAS (University Edition). The level of significance was set at  $P < 0.05$ .

## Results

Prior to assigning pigs to HFD and LFD, no differences in daily feed intake, energy consumption, growth rates, glucose responses or AUC of the glucose response curve were observed among all animals (Figure S1A–E). In addition, no difference was observed in fasting glucose between the two groups ( $P > 0.05$ ).

### Short-term high-fat diet altered growth and feed intake

The effects of 5-week HFD on feed intake, energy consumption and body weight were determined. Pigs fed with HFD consumed more digestible energy ( $\text{kcal d}^{-1}$ ; Figure 1A,  $P < 0.05$ ), even though their feed intake was reduced (Figure 1B,  $P < 0.05$ ), compared with LFD pigs. Consumption of HFD resulted in an average of 5 kg more weight gain than LFD over a 35-d period ( $P < 0.05$ , Figure 1C).

### Short-term high-fat diet increased adiposity and plasma cholesterol in pigs

The adiposity reflected in back fat thickness showed that pigs consuming HFD had significantly increased fat deposition compared with LFD pigs ( $P < 0.05$ , Figure 1D). HFD pigs had significantly elevated concentrations of total cholesterol ( $P < 0.05$ , Figure 1E) and LDL-c ( $P < 0.05$ , Figure 1F) in non-fasting plasma compared with LFD pigs. High-density lipoprotein cholesterol ( $P = 0.83$ , Figure 1G) and triglyceride ( $P = 0.55$ , Figure 1H) concentrations were not different between HFD and LFD.

### Short-term high-fat diet worsened glucose tolerance

Glucose intolerance is commonly seen in obesity (1), therefore, an OGTT was conducted to compare the effects of 4 weeks of HFD and LFD feeding. After overnight

fasting, HFD pigs had a slightly increased blood glucose ( $P = 0.059$ , Figure 2A) compared with LFD, with no difference in fasting plasma insulin ( $P > 0.05$ , Figure 2B). Furthermore, postprandial blood glucose in HFD pigs was significantly higher compared with LFD ( $P < 0.01$ , Figure 2C).

In response to glucose challenge, blood glucose at 60 min was significantly elevated in HFD pigs compared with LFD ( $P < 0.05$ , Figure 2D). The overall glucose response during OGTT, reflected in AUC of the response curve, was significantly higher in HFD compared with LFD ( $P < 0.01$ , Figure 2E). In spite of increased glucose response, pigs consuming HFD had reduced insulin secretion at 30 min ( $P < 0.05$ , Figure 2F) and a trend of lowered AUC of insulin response curve compared with LFD pigs ( $P = 0.09$ , Figure 2G).

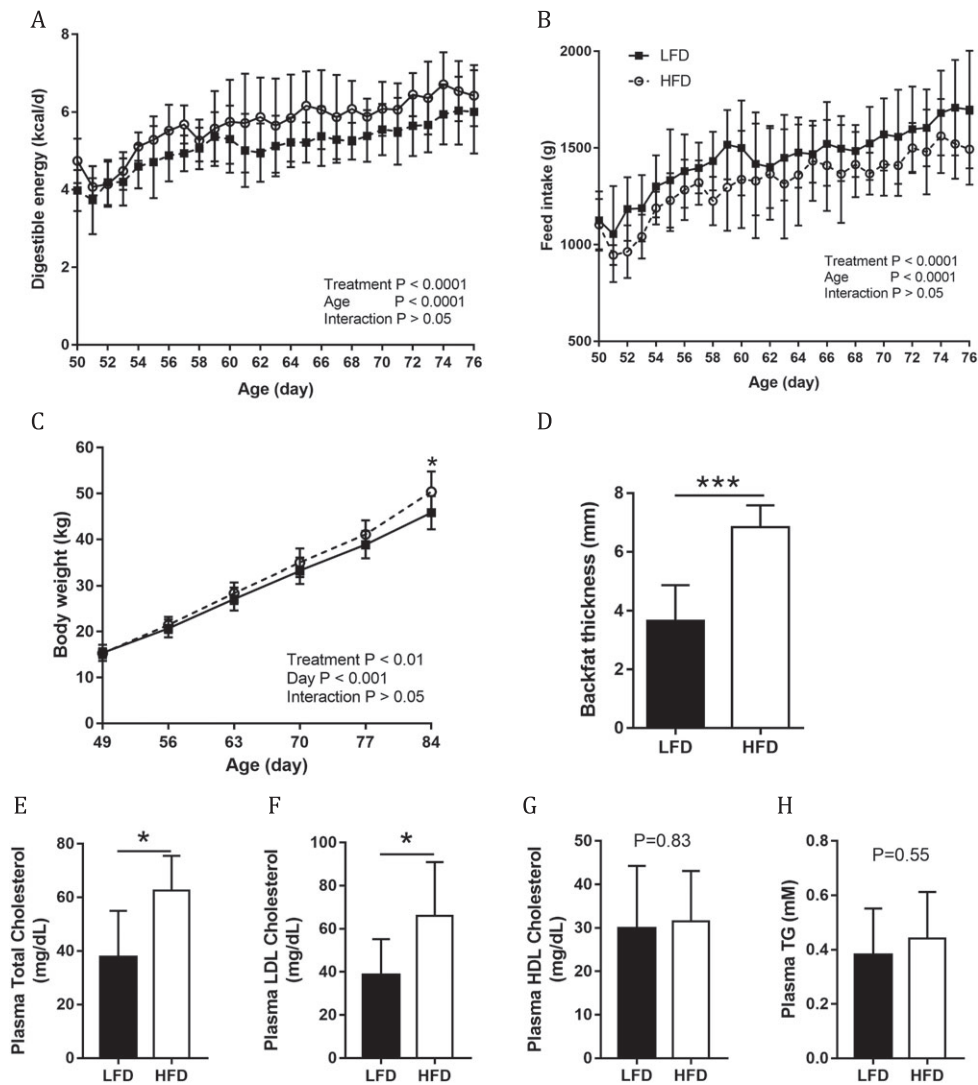
### Short-term high-fat diet led to dysregulated gene expression in back fat

Dyslipidaemia and glucose intolerance are commonly featured with adipose tissue dysfunction (23). Representative genes involved in inflammation and adipogenesis were investigated in back fat. Pigs fed with HFD had increased mRNA levels of toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), TNF (tumour necrosis factor) and transcription factor 7-like 2 (TCF7L2) ( $P < 0.05$ , Figure 3) compared with LFD-fed pigs.

### Short-term high-fat diet altered microbial composition in ileal and proximal colon digesta

Intestinal microbiota from ileum and proximal colon digesta were characterized by sequencing bacterial 16S rRNA gene targeting V3–V4 regions using an Illumina MiSeq platform. The number of sequence reads obtained was 863,965, with an average of 28,000 reads per sample. Samples with less than 1,600 reads were eliminated resulting in  $n = 3/\text{LFD}$  and  $n = 5/\text{HFD}$  for ileum digesta and  $n = 6/\text{LFD}$  and  $n = 6/\text{HFD}$  for proximal colon digesta. OTU clustering (97% similarity) resulted in a total of 180 OTUs in ileal digesta and 821 OTUs in proximal colon digesta.

Proximal colon digesta were predominated by Bacteroidetes and Firmicutes followed by Spirochaetes, Proteobacteria and Tenericutes. Bacterial community structure of the proximal colon changed dramatically in response to HFD, with significantly reduced Bacteroidetes ( $P < 0.05$ , Table 1) and a trend of increased Proteobacteria ( $P = 0.109$ , Table 1). The reduction in Bacteroidetes of HFD pigs was attributable to the reduction in genus *Prevotella* compared with LFD pigs ( $P < 0.05$ , Table 1). In the phylum Firmicutes of pigs

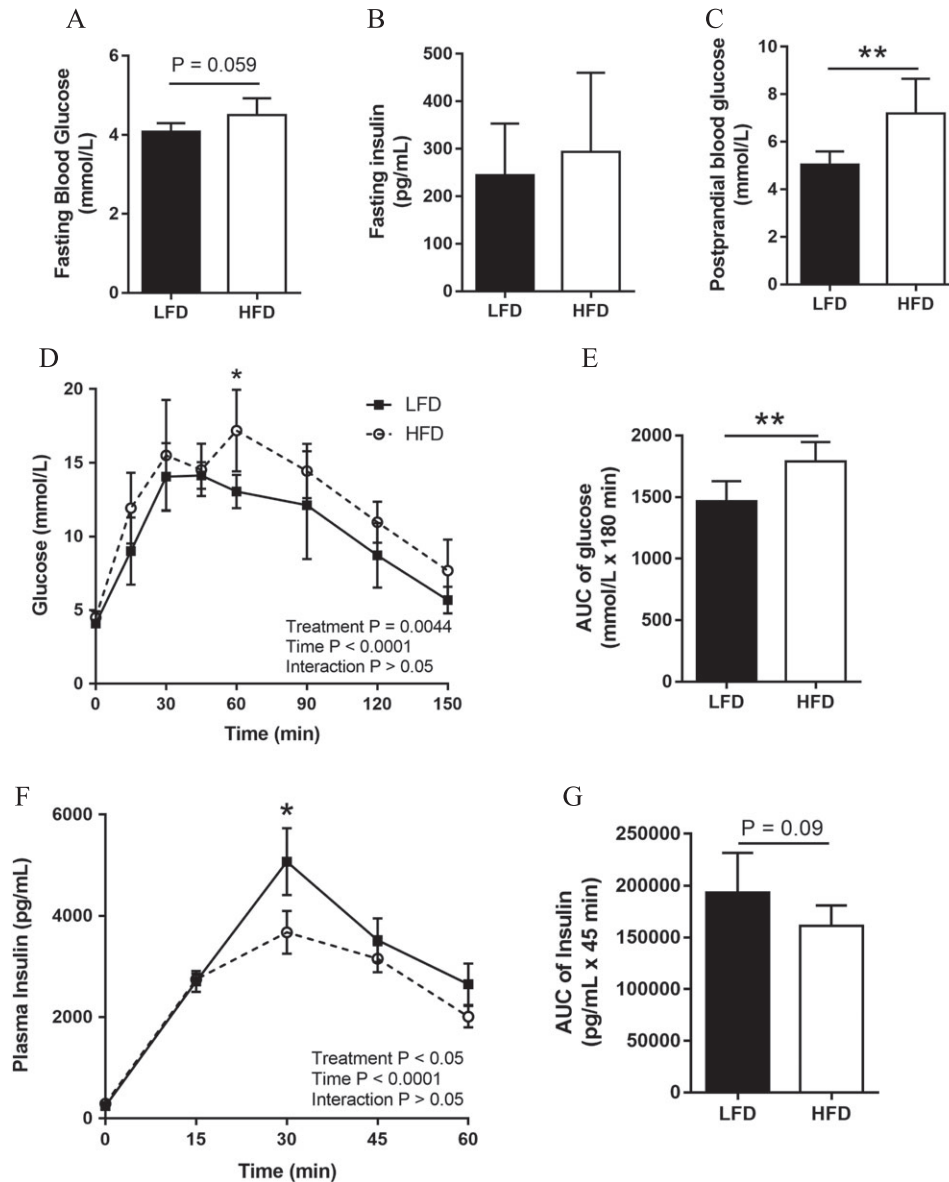


**Figure 1** Short-term high-fat diet (HFD) affected pig growth, feed intake and adiposity. Pigs consuming HFD had reduced feed intake (A), increased digestible energy consumption (B) and subsequent increases in body weight (C) over time in comparison with pigs fed low-fat diet (LFD). HFD pigs subsequently had increased back fat depth (D) compared with LFD pigs. Plasma total cholesterol (E) and low-density lipoprotein cholesterol (F), but not high-density lipoprotein cholesterol (G) and triglycerides (H), were increased in HFD-fed pigs compared with LFD pigs. Data are presented as mean and stdev ( $n = 6$  per group). \* $P < 0.05$ , \*\*\* $P < 0.001$ .

consuming HFD, genera *Lactobacillus*, *Clostridium* and *Selenomonas* increased, while *Mitsuokella* was reduced in comparison with LFD pigs ( $P < 0.05$ , Table 1). HFD pigs also had an increase in *Oxalobacter* and a reduction in *Coriobacteriaceae* ( $P < 0.05$ , Table 1). Due to distinct differences in taxonomic abundances, bacterial community structure between pigs consuming HFD and LFD was distinctly clustered in the principal coordinates analysis plot (ANOSIM  $P < 0.05$ ;  $R = 0.607$ , Figure 4A). Pigs consuming HFD tended to have increased alpha diversity, measured by Inverse Simpson Index ( $P = 0.093$ , Figure 4B).

Consistent with previous reports on microbiota of pigs, Firmicutes were dominant in ileum digesta of both groups, predominated by Clostridiaceae. Firmicutes was followed in abundance by Proteobacteria and Bacteroidetes (Table S3). No statistically significant differences were observed in ileum digesta in microbial composition ( $P > 0.05$ , Table S3) or bacterial diversity ( $P > 0.05$ , Figure S2B) between the two groups. There was no distinction in ileum digesta microbiota community composition observed in the principal coordinates analysis plot on the relative abundance of bacterial OTUs using Bray–Curtis dissimilarity (ANOSIM  $P > 0.05$ , Figure S2A).



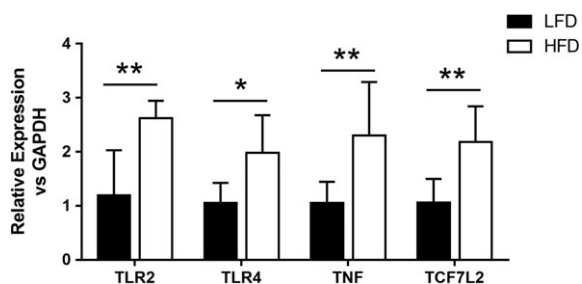


**Figure 2** Short-term high-fat diet (HFD) impaired glucose tolerance. HFD-fed pigs had slightly increased (not statistically significant,  $P = 0.059$ ) fasting glucose concentrations (A), similar fasting insulin concentrations (B) and increased postprandial glucose concentrations ( $P < 0.05$ ) (C) comparing with low-fat diet (LFD) pigs. During oral glucose tolerance test, glucose excursion (D) was different between the two treatments. HFD-fed pigs had increased glucose area under the curve ( $P < 0.05$ ) (E), reduced insulin response to a glucose challenge ( $P < 0.05$ ) (F), and a tendency to have lower insulin area under the curve ( $P = 0.09$ ) (G) in comparison with LFD-fed pigs. Data are presented as mean and stdev ( $n = 6$  per group). \* $P < 0.05$ , \*\* $P < 0.01$ .

### High-fat diet affected ileal short chain fatty acid concentration

High-fat diet-induced changes in gut microbial composition may lead to changes in digesta and plasma SCFA pools. Acetate, butyrate, isovalerate and total SCFA concentrations increased in ileum digesta of HFD pigs

compared with LFD ( $P < 0.05$ , Table 2). With the exception of a minor increase in isobutyrate ( $P < 0.05$ , Table 2) in plasma of pigs consuming HFD versus LFD, no statistically significant differences in SCFA concentrations were observed in proximal colon digesta or plasma of pigs consuming either HFD or LFD ( $P > 0.05$ , Table 2).



**Figure 3** Short-term high-fat diet (HFD) led to dysregulated gene expression in back fat. HFD-fed pigs have increased gene expression of toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), tumour necrosis factor (TNF) and transcription factor 7-like 2 (TCF7L2) in back fat. Data are presented as mean and stdev ( $n = 6$  per group). \* $P < 0.05$ , \*\* $P < 0.01$ . LFD, low-fat diet.

### Alterations in ileal and proximal colon gene expression

To determine whether intestinal functions were affected by diet-induced changes in gut microbiota, gene expression of proteins related to immune activation and innate defence (IL18 [interleukin-18],  $\text{TNF}\alpha$ ,  $\text{REG3}\gamma$ , and  $\text{PPAR}\alpha$  [peroxisome proliferator-activated receptor alpha]), tight junction (ZO2 [zonula occludens 2] and OCLN [occludin]), oxidative stress (HMOX1 [heme oxygenase 1]) and bile acid metabolism (FGF19 [fibroblast growth factor 19]) were measured. Ileal gene expression of IL18 and  $\text{TNF}\alpha$  was significantly increased in pigs consuming HFD versus LFD ( $P < 0.05$ , Table 3). HMOX1 tended to increase ( $P = 0.078$ , Table 3), and  $\text{REG3}\gamma$  tended to reduce ( $P = 0.096$ , Table 3) in ileal tissue from pigs consuming HFD versus LFD. Interestingly, in proximal colon tissue, only IL18 gene expression was significantly increased in pigs consuming LFD versus HFD ( $P < 0.05$ , Table 3), which showed the opposite pattern of that in ileal tissue.

### High-fat diet did not affect plasma lipopolysaccharide and intestinal alkaline phosphatase activity

High-fat diet feeding has been associated with increased translocation of LPS and is thought to be an important mechanism of metabolic inflammation (24). Therefore, circulating LPS concentrations and ileum IAP activity were measured but showed no differences ( $P > 0.05$ , Figures S3A and S3B) between the two groups.

## Discussion

To meet the increasing demand for an appropriate animal model of childhood obesity, the current study aimed to establish a novel animal model in adolescent domestic

**Table 1** Predominant bacteria phyla and genera in digesta from proximal colon of pigs consuming HFD or LFD at postnatal day 84

Phylum	LFD	HFD	$P$ value
Bacteroidetes	62.90 ± 2.81	48.90 ± 8.73	0.004
Firmicutes	31.10 ± 5.64	40.70 ± 11.76	0.150
Spirochaetes	2.20 ± 2.87	3.90 ± 4.50	0.522
Proteobacteria	0.50 ± 0.16	2.30 ± 4.10	0.109
Tenericutes	0.10 ± 0.06	0.10 ± 0.05	0.150
Fibrobacteres	0.10 ± 0.19	0.10 ± 0.05	0.261
Actinobacteria	0.10 ± 0.01	0.10 ± 0.04	0.337
Verrucomicrobia	0.10 ± 0.29	0.00 ± 0.01	0.902
TM7	0.10 ± 0.06	0.10 ± 0.09	1.000
Genus			
Actinobacteria			
<i>Bifidobacterium</i>	ND	0.01 ± 0.01	0.058
Coriobacteriaceae*	0.08 ± 0.04	0.04 ± 0.03	0.037
<i>Collinsella</i>	0.04 ± 0.01	0.05 ± 0.01	0.507
Bacteroidetes			
<i>Prevotella</i>	42.33 ± 1.85	25.19 ± 1.85	0.004
<i>S24-7*</i>	3.54 ± 1.26	5.79 ± 1.98	0.078
Bacteroidales†	4.45 ± 1.15	4.96 ± 2.51	1.000
<i>CF231</i>	2.64 ± 0.85	2.47 ± 0.63	0.749
<i>Parabacteroides</i>	1.68 ± 1.50	1.43 ± 0.66	0.873
Firmicutes			
<i>Phascolarctobacterium</i>	5.79 ± 1.65	6.72 ± 4.01	0.749
<i>Roseburia</i>	3.41 ± 2.07	2.58 ± 2.33	0.150
<i>Anaerovibrio</i>	2.16 ± 0.82	3.21 ± 1.28	0.200
<i>Coprococcus</i>	1.51 ± 1.24	0.80 ± 0.55	0.262
<i>Oscillospira</i>	1.36 ± 0.46	1.70 ± 0.76	0.873
<i>Ruminococcus</i>	0.92 ± 0.41	0.62 ± 0.27	0.109
<i>Lactobacillus</i>	0.64 ± 0.61	2.93 ± 0.56	0.004
<i>Blautia</i>	0.52 ± 0.21	0.62 ± 0.43	0.873
<i>Lachnospira</i>	0.51 ± 0.50	0.36 ± 0.09	0.873
<i>p-75-a5</i>	0.27 ± 0.06	0.24 ± 0.24	0.150
<i>Mitsuokella</i>	0.14 ± 0.12	0.02 ± 0.06	0.020
<i>Clostridium</i>	0.07 ± 0.04	2.31 ± 4.75	0.010
<i>Selenomonas</i>	0.01 ± 0.02	0.16 ± 0.20	0.009
Spirochaetes			
<i>Treponema</i>	2.06 ± 2.76	3.82 ± 4.43	0.522
Proteobacteria			
<i>Succinivibrio</i>	0.14 ± 0.08	1.86 ± 4.15	0.423
<i>Oxalobacter</i>	0.01 ± 0.01	0.09 ± 0.07	0.028
Verrucomicrobia			
<i>Akkermansia</i>	0.12 ± 0.29	ND	0.902

Data are presented as means with stdev,  $n = 6$  per each group.

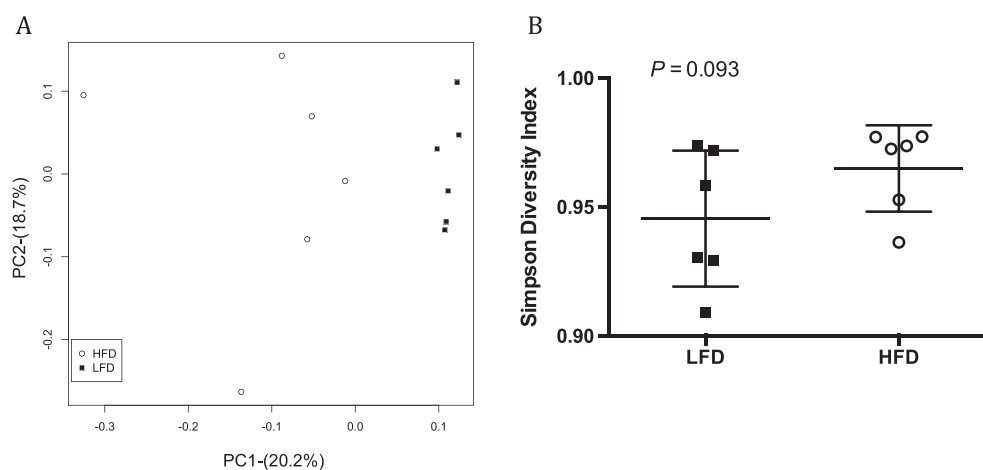
\*Unclassified family.

†Unclassified order.

HFD, high-fat diet; LFD, low-fat diet; ND, not detected.

pigs by HFD feeding. This model proved to be sufficient to observe features of childhood obesity including alterations in body weight, glucose tolerance, cholesterol and lipid profiles, inflammatory markers and microbial composition.

Adolescent pigs fed a 5-week HFD developed an obese phenotype, having increased body weight and 46% greater back-fat depth compared with pigs



**Figure 4** Short-term high-fat diet (HFD) altered microbial composition in proximal colon digesta. (A) Based on Bray–Curtis dissimilarity showed, pigs consuming HFD versus low-fat diet (LFD) had distinct proximal colon bacterial communities as determined with ANOSIM ( $P = 0.002$ ;  $R = 0.607$ ;  $n = 6$ /HFD and LFD). Alpha diversity in proximal colon microbiota (B) measured with inverse Simpson diversity had a tendency to be higher in pigs consuming HFD in comparison with LFD ( $P = 0.093$ ).

**Table 2** Short chain fatty acid concentrations in plasma and digesta of ileum and proximal colon from pigs fed HFD or LFD at postnatal day 84

	SCFA	HFD	LFD	$P$ value*
Ileal digesta, $\mu\text{mol g}^{-1}$	Acetate	$16.1 \pm 3.12$	$8.5 \pm 4.59$	0.020
	Propionate	$0.5 \pm 0.24$	$0.7 \pm 0.66$	0.364
	Butyrate	$1.1 \pm 0.48$	$0.4 \pm 0.41$	0.039
	Isovalerate	$0.5 \pm 0.21$	$0.1 \pm 0.15$	0.038
	Isobutyrate	$0.2 \pm 0.13$	$0.2 \pm 0.28$	0.599
	Total	$18.5 \pm 0.02$	$10.0 \pm 0.02$	0.039
	Proximal colon digesta, $\mu\text{mol g}^{-1}$	Acetate	$36.6 \pm 6.46$	$39.3 \pm 5.86$
Propionate		$14.3 \pm 2.18$	$14.7 \pm 2.27$	0.873
Butyrate		$8.3 \pm 0.87$	$7.7 \pm 1.10$	0.149
Isovalerate		$0.8 \pm 0.53$	$0.5 \pm 0.14$	0.200
Isobutyrate		$0.7 \pm 0.27$	$0.6 \pm 0.06$	0.200
Plasma, $\mu\text{mol mL}^{-1}$	Total	$61.4 \pm 0.04$	$63.4 \pm 0.01$	0.631
	Acetate	$7.2 \pm 1.07$	$7.0 \pm 1.05$	0.698
	Propionate	$3.0 \pm 0.58$	$3.2 \pm 0.43$	0.476
	Butyrate	ND	ND	--
	Isovalerate	ND	ND	--
	Isobutyrate	$0.5 \pm 0.03$	$0.4 \pm 0.06$	0.0009
	Total	$11.1 \pm 1.69$	$10.9 \pm 1.49$	0.813

Data are presented as means with stdev.

\* $P$  values are for Kruskal–Wallis comparison between HFD and LFD ( $n = 6$ /HFD in ileum and  $n = 3$ /LFD in ileum;  $n = 6$ /HFD and LFD in plasma and proximal colon).

SCFA, short chain fatty acid; HFD, high fat diet; LFD, low fat diet; ND, not detected; --, not calculated.

consuming a LFD, which is in agreement with previous findings (25). Back fat thickness is commonly used as a measure of obesity in pigs (26,27), and increased fat

**Table 3** Relative gene expression in ileum and proximal colon of pigs fed HFD or LFD at postnatal day 84.

	Gene	HFD	LFD	$P$ value*	
Ileum	REG3 $\gamma$	$0.26 \pm 0.52$	$1.27 \pm 1.11$	0.096	
	IL18	$3.06 \pm 0.92$	$1.12 \pm 0.64$	0.002	
	PPAR $\alpha$	$1.33 \pm 0.26$	$1.13 \pm 0.55$	0.452	
	ZO2	$1.48 \pm 0.26$	$1.16 \pm 0.64$	0.277	
	HMOX1	$1.64 \pm 0.74$	$1.02 \pm 0.21$	0.078	
	TNF	$2.76 \pm 1.66$	$1.07 \pm 0.44$	0.036	
	FGF19	$2.00 \pm 2.59$	$1.38 \pm 1.10$	0.601	
	OCLN	$1.35 \pm 0.23$	$1.26 \pm 0.85$	0.806	
	Proximal colon	REG3 $\gamma$	$0.67 \pm 0.68$	$1.42 \pm 1.50$	0.293
		IL18	$0.49 \pm 0.14$	$1.07 \pm 1.07$	0.010
PPAR $\alpha$		$1.06 \pm 0.12$	$1.03 \pm 0.31$	0.872	
ZO2		$0.92 \pm 0.21$	$1.05 \pm 0.33$	0.455	
HMOX1		$1.11 \pm 0.41$	$1.12 \pm 0.57$	0.971	
TNF		$1.30 \pm 0.46$	$1.10 \pm 0.52$	0.492	
FGF19		$6.02 \pm 7.96$	$1.33 \pm 1.33$	0.196	
OCLN	$0.88 \pm 0.14$	$1.05 \pm 0.36$	0.311		

Data are presented as means with stdev. Detailed gene information is listed in Table S2.

\* $P$  values are for Kruskal–Wallis comparison between HFD and LFD ( $n = 6$  per each group)

FGF19, fibroblast growth factor 19; HFD, high-fat diet; HMOX1, heme oxygenase 1; IL18, interleukin-18; LFD, low-fat diet; ND, not detected; PPAR $\alpha$ , peroxisome proliferator-activated receptor alpha; TNF, tumour necrosis factor.

deposition is associated with dysregulated gene expression (such as TCF7L2 and leptin) that are high risk susceptibility genes for type 2 diabetes and obesity (28,29).



In accordance, TCF7L2 gene expression in back fat was increased in HFD-fed pigs at PND84. HFD feeding is closely related with endotoxemia which is proposed to be a primary factor initiating obesity (24). However, short-term HFD feeding did not change plasma LPS concentrations in this study. Nonetheless, TLR4 gene expression was increased in back fat from HFD pigs, as well as TLR2 and TNF $\alpha$ . In adipocytes, HFD feeding increases the expression of TLR2 and TNF $\alpha$  and the number of cells expressing TLR2/TNF $\alpha$  (30), and activation of TLR4 induces TLR2 synthesis and TNF $\alpha$  production (31). Also, TNF $\alpha$  inhibits adipogenesis mediated by a  $\beta$ -catenin/TCF7L2-dependent pathway (32). Therefore, the increased gene expression observed is suggestive of activation of inflammation in back fat, indicating adipocyte dysfunction that need to be further confirmed.

Furthermore, HFD pigs also exhibited increased circulating total cholesterol and LDL-c, which signifies the development of dyslipidaemia. All pigs in the current study started with equal body weight and feed intakes making the obese phenotype observed in HFD-fed pigs at PND 84 attributable to increased energy consumption, albeit with reduced voluntary feed intake, which was previously observed (33).

In previous studies, feeding a HFD (~20%, w/w) to pigs for 7 (15,25) or 11 weeks (14) was insufficient to observe changes in weight, but these studies neglected to characterize glucose tolerance, which is often impaired with obesity. Whereas, the present study found 5-week HFD feeding was adequate to induce obesity, including impaired glucose tolerance. The success of this study may be due to the early exposure of pigs to HFD on PND 49 versus 3 months of age in previous research. This indicates that early life exposure to HFD might have a greater influence on the development of metabolic dysfunction. In response to a standard glucose challenge, HFD-fed pigs had a significantly elevated glucose response compared with those fed with LFD during OGTT coupled with reduced insulin secretion suggesting pigs fed HFD had insufficient insulin secretion to promote glucose uptake, resulting in impaired glucose tolerance. Correspondingly, HFD pigs had significantly higher post-prandial glucose at 180 min after glucose challenge, as well as an increasing trend in fasting blood glucose compared with LFD, even though no difference in fasting plasma insulin concentrations were observed between the two diet groups.

In rats, a 12-week HFD led to ileum inflammation with increased TLR4 activation and reduced IAP activity (34). In the present study, ileum IAP activity was not different between the two diets. However, increased gene expression of TNF $\alpha$  was detected in the ileum of pigs consuming

HFD versus LFD, indicating the possibility of HFD-induced upregulation of inflammatory response in the ileum. In addition, HMOX1, a proinflammatory biomarker found in obesity and metabolic disease (35), tended to increase in expression in the ileum of HFD pigs. Interestingly, gene expression of IL18 was upregulated in the ileum. IL18 is an anti-obese/inflammatory cytokine secreted in the progression of obesity as a negative feedback signal, and paracrine IL18 secretion is able to decrease adiposity to balance fat accumulation in response to HFD in mice (36). The increase in ileum IL18 gene expression is suggestive of negative feedback of pigs against HFD-induced obesity. On the other hand, the production of IL18 can be affected by gut microbiota (37). The differential IL18 expression between proximal colon and ileum in HFD pigs might be a result of distinct microbial composition of the two sites, which need to be confirmed by further analysis. Ileal inflammation has also been associated with altered tight junction permeability (34). However, ileal gene expression of ZO2 and OCLN did not differ between treatments, suggesting tight junctions might not be affected. This could be the result of increased butyrate concentrations in the ileum of pigs fed HFD, as microbial butyrate can restore the tight junction barrier (38).

High-fat feeding-induced changes in abundance or metabolic activity of specific microbial taxa have previously been established. Reduced abundance of Bacteroidetes found in the current study is in agreement with previous findings in obese Ossabaw and Banna minipigs fed with high energy density diets(39,40) and recapitulates findings in obese mice (41,42) and humans (13). In contrast, a recent study in conventional pigs found increased Bacteroidetes (*Bacteroides* group and *Prevotella* spp.) in both cecum and colon of pigs fed a HFD (25). In obese Ossabaw minipigs, reduced abundance of genera *Prevotella* and increased abundance of *Clostridium* were observed in the colon compared with their lean counterparts, analogous to findings in the current study (40).

In the present study, pigs fed with HFD had increased ileal concentrations of acetate, butyrate, isovalerate and total SCFA. Evidence has recently emerged showing that HFD (43) and obesity (41) are linked with increased acetate concentrations, in agreement with the current findings. Furthermore, HFD-induced increases in acetate production activate the parasympathetic nervous system, stimulating ghrelin and glucose-stimulated insulin secretion, hyperphagia, obesity and insulin resistance (43). Consistent with this idea, HFD pigs in the current study consumed substantially more calories per day than LFD pigs, developed greater adiposity and became glucose intolerant.

## Conclusion

In the current study, it was demonstrated that adolescent domestic pigs are a suitable alternative to rodent models to study the pathophysiology of childhood obesity. They develop obesity after 5-week HFD feeding with features of adiposity, dyslipidaemia and glucose intolerance. In addition, changes in the gut microbiota and intestinal gene expression resembled findings in other obesity models. This model can facilitate the development of strategies for prevention and treatment of childhood obesity.

## Acknowledgements

We are grateful to the Swine Research and Technology Centre staff for their support in animal experiments. We thank Lisa Nikolai and Samuel Yue (The Chromatography Facility, Department of Agriculture, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada) for measuring short chain fatty acids.

## Funding

This work was supported by operating grants from the CIHR to B. P. W. and C. B. C. B. P. W. is supported by the Canada Research Chair program. T. J. is supported by Alberta Innovates Technology Futures scholarship. E. M. received a scholarship from Natural Sciences and Engineering Research Council of Canada.

## Author Contributions

J. F. and K. Y. contributed equally to this work. J. F. and K. Y. performed the experiments, analysed the data and wrote the manuscript. J. L., E. M., T. J. and C. S. A. performed the experiments, analysed the data and edited the manuscript. C. B. C. helped conceive the project and edited the manuscript. B. P. W. conceived the project, wrote the manuscript and is the guarantor of this publication. All authors had final approval of the submitted and published versions.

## Conflict of Interest Statement

The authors declare no conflict of interest.

## References

- Bray GA. Obesity: the disease. *J Med Chem* 2006; **49**: 4001–4007.
- Ogden CL, Carroll MD, Fryar CD, Flegal KM. Prevalence of obesity among adults and youth: United States, 2011–2014. 2015.
- Roberts KC, Shields M, de Groh M, Aziz A, Gilbert J-A. Overweight and obesity in children and adolescents: results from the 2009 to 2011 Canadian Health Measures Survey. 2012.
- Commission on Ending Childhood Obesity WHO. Report of the Commission on Ending Childhood Obesity. Geneva, Switzerland; 2016.
- Lai M, Chandrasekera PC, Barnard ND. You are what you eat, or are you? The challenges of translating high-fat-fed rodents to human obesity and diabetes. *Nutr Diabetes* 2014; **4**: e135.
- Spurlock ME, Gabler NK. The development of porcine models of obesity and the metabolic syndrome. *The Journal of Nutrition* 2008; **138**: 397–402.
- Heinritz SN, Mosenthin R, Weiss E. Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. *Nutr Res Rev* 2013; **26**: 191–209.
- Xi S, Yin W, Wang Z, et al. A minipig model of high-fat/high-sucrose diet-induced diabetes and atherosclerosis. *Int J Exp Pathol* 2004; **85**: 223–231.
- Neeb ZP, Edwards JM, Alloosh M, Long X, Mokelke EA, Sturek M. Metabolic syndrome and coronary artery disease in Ossabaw compared with Yucatan swine. *Comp Med* 2010; **60**: 300–315.
- Saari A, Virta LJ, Sankilampi U, Dunkel L, Saxen H. Antibiotic exposure in infancy and risk of being overweight in the first 24 months of life. *Pediatrics* 2015; **135**: 617–626.
- Li J, Yang K, Ju T, et al. Early life antibiotic exposure affects pancreatic islet development and metabolic regulation. *Sci Rep* 2017; **7**: 41778.
- Ferrer M, Ruiz A, Lanza F, et al. Microbiota from the distal guts of lean and obese adolescents exhibit partial functional redundancy besides clear differences in community structure. *Environ Microbiol* 2013; **15**: 211–226.
- Tumbaugh PJ, Hamady M, Yatsunenko T, et al. A core gut microbiome in obese and lean twins. *Nature* 2009; **457**: 480–484.
- Pedersen R, Andersen AD, Hermann-Bank ML, Stagsted J, Boye M. The effect of high-fat diet on the composition of the gut microbiota in cloned and non-cloned pigs of lean and obese phenotype. *Gut Microbes* 2013; **4**: 371–381.
- Heinritz SN, Weiss E, Eklund M, et al. Intestinal microbiota and microbial metabolites are changed in a pig model fed a high-fat/low-fiber or a low-fat/high-fiber diet. *PLoS One* 2016; **11**: 1–21.
- Cirera S. Highly efficient method for isolation of total RNA from adipose tissue. *BMC Res Notes* 2013; **6**: 472.
- Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010; **7**: 335–336.
- Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinf* 2012; **13**: 31.
- Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010; **26**: 2460–2461.
- Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007; **73**: 5261–5267.
- Navas-Molina JA, Peralta-Sánchez JM, González A, et al. Advancing our understanding of the human microbiome using QIIME. *Methods Enzymol* 2013; **531**: 371–444.
- Colwell RK, Chao A, Gotelli NJ, et al. Models and estimators linking individual-based and sample-based rarefaction, extrapolation and comparison of assemblages. *J Plant Ecol* 2012; **5**: 3–21.
- Schäffler A, Schölmerich J, Salzberger B. Adipose tissue as an immunological organ: toll-like receptors, C1q/TNFs and CTRPs. *Trends Immunol* 2007; **28**: 393–399.
- Cani PD, Amar J, Iglesias MA, et al. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 2007; **56**: 1761–1772.

25. Heinritz S, Weiss E, Eklund M, et al. Impact of a high-fat or high-fiber diet on intestinal microbiota and metabolic markers in a pig model. *Nutrients* 2016; **8**: 317.
26. Camacho-Rea C, Arechavaleta-Velasco ME, Gutiérrez CG, et al. Evaluation of metabolic, endocrine and growth features in the Mexican hairless pig to determinate its potencial as model for obesity in comparison with commercial pigs. *Ital J Anim Sci* 2010; **9**: e84.
27. Dyson MC, Alloosh M, Vuchetich JP, Mokolke EA, Sturek M. Components of metabolic syndrome and coronary artery disease in female Ossabaw swine fed excess atherogenic diet. *Comp Med* 2006; **56**: 35–45.
28. Robert C, Palin MF, Coulombe N, et al. Backfat thickness in pigs is positively associated with leptin mRNA levels. *Can J Anim Sci* 1998; **78**: 473–482.
29. Du Z-Q, Fan B, Zhao X, Amoako R, Rothschild MF. Association analyses between type 2 diabetes genes and obesity traits in pigs. *Obesity* 2009; **17**: 323–329.
30. Murakami K, Bujo H, Unoki H, Saito Y. High fat intake induces a population of adipocytes to co-express TLR2 and TNF $\alpha$  in mice with insulin resistance. *Biochem Biophys Res Commun* 2007; **354**: 727–734.
31. Lin Y, Lee H, Berg AH, Lisanti MP, Shapiro L, Scherer PE. The lipopolysaccharide-activated toll-like receptor (tlr)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. *J Biol Chem* 2000; **275**: 24255–24263.
32. Cawthorn WP, Heyd F, Hegyi K, Sethi JK. Tumour necrosis factor- $\alpha$  inhibits adipogenesis via a  $\beta$ -catenin/TCF4(TCF7L2)-dependent pathway. *Cell Death Differ* 2007; **14**: 1361–1373.
33. Liu J, He J, Yu J, et al. Birth weight alters the response to postnatal high-fat diet-induced changes in meat quality traits and skeletal muscle proteome of pigs. *Br J Nutr* 2014; **111**: 1738–1747.
34. de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. *AJP Gastrointest Liver Physiol* 2010; **299**: G440–G448.
35. Jais A, Einwallner E, Sharif O, et al. Heme oxygenase-1 drives metaflammation and insulin resistance in mouse and man. *Cell* 2014; **158**: 25–40.
36. Murphy AJ, Kraakman MJ, Kammoun HL, et al. IL-18 production from the NLRP1 inflammasome prevents obesity and metabolic syndrome. *Cell Metab* 2016; **23**: 155–164.
37. Davis BK, Wen H, JP-Y T. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol* 2011; **29**: 707–735.
38. Kelly CJ, Zheng L, Campbell EL, et al. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. *Cell Host Microbe* 2015; **17**: 662–671.
39. Guo X, Xia X, Tang R, Zhou J, Zhao H, Wang K. Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Letf Appl Microbiol* 2008; **47**: 367–373.
40. Pedersen R, Ingerslev H-C, Sturek M, et al. Characterisation of gut microbiota in Ossabaw and Göttingen minipigs as models of obesity and metabolic syndrome cotter PD. *PLoS One* 2013; **8**: e56612.
41. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JL. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006; **444**: 1027–1131.
42. Serino M, Luche E, Gres S, et al. Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota. *Gut* 2012; **61**: 543–553.
43. Perry RJ, Peng L, Barry NA, et al. Acetate mediates a microbiome–brain– $\beta$ -cell axis to promote metabolic syndrome. *Nature* 2016; **534**: 213–217.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Experimental diet ingredient composition

**Table S2.** Primer sequences for quantitative RT-PCR analysis

**Table S3.** Predominant bacteria phyla and genera in ileal digesta of pigs consuming HFD or LFD at PND 84

**Figure S1.** Baseline measurements before assigning treatment diets

**Figure S2.** Microbial composition in ileal digesta

**Figure S3.** Plasma LPS concentrations and intestinal alkaline phosphatase activity