10-1-2020

# Abundance of the species Clostridium butyricum in the gut microbiota contributes to differences in obesity phenotype in outbred Sprague-Dawley CD rats

Diana N. Obanda
*University of Maryland*

Claudia Husseneder
*LSU Agricultural Center*

Anne M. Raggio
*LSU Agricultural Center*

Ryan Page
*LSU Agricultural Center*

Brian Marx
*Louisiana State University*

*See next page for additional authors*



## Recommended Citation

Obanda, D., Husseneder, C., Raggio, A., Page, R., Marx, B., Stout, R., Guice, J., Coulon, D., & Keenan, M. (2020). Abundance of the species Clostridium butyricum in the gut microbiota contributes to differences in obesity phenotype in outbred Sprague-Dawley CD rats. *Nutrition, 78* https://doi.org/10.1016/j.nut.2020.110893

HHS Public Access

Author manuscript

*Nutrition*. Author manuscript; available in PMC 2022 February 16.

Published in final edited form as:

*Nutrition*. 2020 October ; 78: 110893. doi:10.1016/j.nut.2020.110893.

# Abundance of the species *Clostridium butyricum* in the gut microbiota contributes to differences in obesity phenotype in outbred Sprague-Dawley CD rats

**Diana Obanda**[1], **Claudia Husseneder**[2], **Anne M. Raggio**[3], **Ryan Page**[3], **Brian Marx**[4], **Rhett W Stout**[5], **Justin Guice**[6], **Diana Coulon**[3], **Michael J. Keenan**[3]

[1]University of Maryland, Department of Nutrition and Food Sciences, College Park, MD, USA;

[2]Entomology, LSU AgCenter, Baton Rouge, LA, USA;

[3]Nutrition and Food Sciences/Animal Sciences, LSU AgCenter, Baton Rouge, LA, USA;

[4]Experimental Statistics, LSU, Baton Rouge, LA;

[5]Department of Pathobiological Sciences, LSU School of Veterinary Medicine, LSU A & M, Baton Rouge, LA, USA;

[6]BIO-CAT, 9117 Three Notch Road, Troy, VA, USA

## Abstract

**Scope and methods—**Gut microbiota profiles contribute to differences in obesity phenotype. We examined the abundance of total gut bacteria and the species *Clostridium butyricum* in relation to the propensity of obesity prone and obesity resistant outbred Sprague-Dawley rats to accumulate abdominal fat. We further examined effects of dietary fat, resistant starch (RS), and a microbiota transplant on obesity phenotype and abundance of *C. butyricum*.

**Results—**Before inclusion of dietary RS, obesity resistant (OR) rats had higher amounts of total bacteria, and *C. butyricum* compared to obesity prone (OP) rats ($P<0.005$ in study I, $P<0.0001$ in study II). A high fat diet (HF) lowered *C. butyricum* levels while RS had no effect. Dietary RS elicited robust fermentation and increased total bacteria only in OP rats. In preparation for the transplant, antibiotics were administered to recipient rats. Four weeks thereafter, total bacteria

Correspondence: Diana Obanda. Skinner Building Room 0112, University of Maryland, College Park, MD. dobanda@umd.edu.

Author Contributions/Credit Author Statement

Diana Obanda designed the study, performed the two animal studies, analyzed the tissues, wrote the draft manuscript and edited it; Claudia Husseneder designed the study and edited the manuscript; Anne Raggio performed part of the animal studies and tissue analysis; Ryan Page performed part of the animal studies; Brian Marx advised on and performed data analysis; Rhett Stout performed part of the animal studies; Justin Guice performed part of the animal studies and data analysis; Diana Coulon formulated the diets and performed part of the animal studies; Michel Keenan designed the study, performed the two animal studies work, reviewed and edited the manuscript and acquired the funding.



Disclosure

Michael J. Keenan has received research funding and gifts of starch products from Ingredion Incorporated. The other authors declared no conflict of interest.

levels were restored but, *C. butyricum* levels were not. The transplant between the two phenotypes had no effect on abundance of *C. butyricum* and obesity phenotype.

**Conclusions—**While *C. butyricum* is a known saccharolytic, its proliferation is not enhanced by fermentation of resistant starch. *C. butyricum* maybe one of the species that constitute a core microbiota involved in energy storage and metabolism through mechanisms that are not yet known.

### Keywords

*Clostridium butyricum* ; Diet; Fat accumulation; Microbiota; Resistant Starch

## INTRODUCTION

Studies examining the composition of intestinal microbiota in relation to obesity have consistently shown an increase in the relative abundance of phylum *Firmicutes* and a reduction in phylum *Bacteroidetes* in the microbiota of obese and lean mice and humans [1,2]. However, Firmicutes contains more than 270 genera and Bacteroidetes more than 20 genera. Differences at phylum level do not pinpoint specific bacteria associated with obesity. Causal implication of specific bacterial species to the obese phenotype has not been shown and different species can be part of the same functional group performing similar metabolic tasks. It is possible that variations in specific bacterial species rather than phylum level changes are more important.

For this study, we used outbred Sprague-Dawley CD rats which portray a polygenic pattern of inheritance that mimics the human obese phenotype. The strain portrays two phenotypes: the obesity-resistant rats (OR) fed a high fat (HF) diet retain a leaner frame while the obesity-prone (OP) rapidly gain weight within 4 weeks when fed the same diet [3,4]. Both phenotypes have a fully functioning leptin receptor with no endocrine disturbances like those observed in db/db mice or the Zucker diabetic fatty rats. It is therefore an appropriate animal model for studying mechanisms and mitigation strategies for human obesity. We hypothesized that differences in the obesity phenotype may be attributed to the microbiota composition, ability of the microbiota to ferment dietary fiber and that the abundance of specific species correlates with propensity to accumulate fat. Herein we report the findings from two independent studies focused on the abundance of the species *C. butyricum* in relation to diet-induced abdominal fat accumulation.

Dietary fibers that physiologically impact body weight through production of short chain fatty acids include resistant starch (RS), beta-glucan, oligosaccharides and pectin [5,6]. Based on previous work on RS in our laboratory we focused on comparing RS fermentation in OP and OR rats. We also focused on *C. butyricum* because when given as a probiotic, it protects mice from developing high fat diet induced obesity, intestinal inflammation and enhances immune function and stability [7,8]. Furthermore, this species selectively produces butyrate which imparts anti-inflammatory properties [9,10]. We further hypothesized that robust butyrate production on provision of RS was one of the mechanisms by which the OR rats retain a leaner frame and a more favorable metabolic profile compared to the OP rats. Using a targeted culture independent DNA-based method, we studied abundance of

total bacteria and *C. butyricum* in relation to the obesity phenotype and to diet over 8 weeks in study I. In study II, we studied the effect of a microbiota transplant in transmitting the donor phenotype and abundance of *C. butyricum* between the OP and OR rats. Prior to the transplant, we tested the efficacy of two antibiotic formulations in reducing the gut bacterial load of recipient rats. Antibiotics are supposed to create a niche for the probiotic bacteria to establish effectively [11]. We also included MiraLAX® an over the counter colonoscopy preparation that has been shown to knock down bacterial levels in the lower gut [12] · We compared the efficacy of MiraLAX® to that of the antibiotics as an alternative in creating a niche for the probiotic bacteria. We report on the effects of diet, antibiotics, MiraLAX® and the transplant on total bacteria and the abundance of *C. butyricum* in relation to the obesity phenotype.

## MATERIALS AND METHODS

### Animals

The two sequential studies approved by the Louisiana State University Institutional Animal Care and Use Committee as 15–109 utilized 3 and 4-week-old male OP and OR rats (Charles River Laboratories) singly housed with a 12:12-hour light/dark cycle. Table 1 shows acronyms of treatments. Sample size of n=6 for each treatment was based on a power analysis in a previous resistant starch fermentation study in rats that showed 6 to be sufficient to generate statistically significant results at a power of 94.8% [14]. To attain a statistical power of 0.90, 6 samples are needed for a one tailed test and 8 samples for a two tailed test.

### Diets and Feeding for both study I and II

The composition and energy value of the modified AIN-93M diets are shown in Table 2. The LFRS and HFRS diets were formulated to contain 20% RS and were isocaloric to the LF and HF diets respectively. Food and water were provided *ad libitum*. Body weight and food intake were determined twice a week. Rats were stratified into dietary groups (study I) or knock down groups (study II) based on body weight and the homeostatic model of assessment of insulin resistance index (HOMA-IR) calculated as shown before [13].

### Plans for study I and II

Details of study I procedures have been reported [13], but briefly, both OP and OR rats were fed either a HF (22 rats) or LF diet (10 rats) for 4 weeks (phase 1). Four OP and four OR rats were then euthanized from both diet groups. In phase 2, the 6 OP and 6 OR rats continued on the LF diet, 18 OP and 18 OR rats from the HF group were split randomly into three groups of 6. One group continued on HF (HF-HF), the second was switched to LF (HF-LF), and the third was switched to LF with 20% RS (HF-LFRS) (Figure 1). In study II, all OP (28 rats) and OR (28 rats) were fed a HF for 4 weeks (phase 1). Four OP and four OR rats were then euthanized to determine body fat accumulation and aseptically collect cecal contents as inoculation probiotic for the transplant. Cecal contents were diluted 1:10 with sterile 15% glycerol/PBS solution. Cecal contents from the four OP rats were pooled into one sample and those from the four OR rats were also pooled into one sample.

### Knock down of microbiota and bacterial transplant

The remaining 24 rats for each phenotype were divided into 4 sub-groups of 6. Fresh fecal samples were collected prior to the knock down treatment. The following day knock down was performed: sub-group 1 was given neomycin + ampicillin (NA), both broad-spectrum antibiotics, sub-group 2 was given vancomycin + meropenem (VM), one narrow-spectrum and one broad-spectrum antibiotic, respectively. A dose of 50 mg/kg/day each for Vancomycin and meropenem was given in a single gavage for 3 days. Neomycin was given at a dose of 55 mg/kg/day and ampicillin was given at 110 mg/kg/day in a single gavage for 3 days. Sub-group 3 received a water gavage for two days. On the third day, MiraLAX® was given in two gavages three hours apart at ~8:30, and 11:30 am. Directions for MiraLAX® for humans are 17 g dissolved in 4–8 ounces of fluid for a 150-lb. person. This translated to a dose of 63 mg in 437 ul for a 250g rat. Sub-group 4 was given water by gavage as control treatment. After knock down treatments, rats had free access to the HF diet and water for 2 days before fresh fecal samples were collected.

### Bacterial transplant

On the 3rd day after knock down, one ml aliquots of cecal inoculum from OR rats were transplanted into OP rats and contents from donor OP rats were transplanted into the OR rats by oral gavage over three days. Control rats previously given water as control again received 1ml water as transplant control. After all transplants, the rats were switched to a HF diet containing RS (HF-RS) for 4 weeks (Phase 2).

### Collection and analysis of Tissues

Serum, fat pads, cecal contents and emboweled body weight (EBW) were collected and/or determined as shown before [13]. Short chain fatty acids in cecal contents were determined by gas chromatography as described before [13].

### Bacterial DNA extraction and primer selection

DNA was extracted from 200mg of cecal contents using the QIAamp DNA Fast Stool Mini Kit (Qiagen, Valencia, California). Primers for 16S rRNA gene were F:5′ACGTCRTCCMCNCCTTCCTC3′ and R:5′GTGSTGCAYGGYYGTCGTCA3′, as reported by Belenguer et al. [13]. Primers for *C. butyricum* were F: GTGCCGCCGCTAACGCATTAAGTAT 3’ and R: CCATGCACCACCTGTCTTCCTGCC 3’ as reported by Bartosch et al. [16]. Fermentation levels of RS were assessed by amounts of the archaea *M. smithii*. Primers for *M. smithii* were F:5’CCGGGTATCTAATCCGGTTC3’ and R:5’CTCCCAGGGTAGAGGTGAAA3’ as reported by Dridi et al. [17]. The Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed June 25th 2017 was used to verify *in silico* that primers for *C. butyricum* and *M. smithii* were specific to these species and were 100% identical to the corresponding regions of the 213 and 123bp double-stranded amplicons respectively. *In silico* results for the universal 16S rRNA primers matched a broad range of bacteria.

### Preparation of DNA standards, Quantitative real-time PCR and calculation of gene copies

Genomic DNA from pooled samples from each treatment was used as PCR template to prepare amplicon DNA with specific gene sequences and lengths (213 bp for *C. butyricum*; 123 bp for *M. smithii* and 147 bp for universal 16S rRNA genes). Annealing temperatures were 59°C for *C. butyricum*, 52°C for *M. smithii* and 60°C for 16S rRNA gene. Standard curves ranging from $10^8$ to $10^2$ copies per microliter were generated by serial 10-fold dilutions of amplicon DNA. The SYBR Green qPCR assay was used to quantify total bacteria, *C. butyricum* and *M. smithii* (ABI Prism 7900HT Sequence Detection System, Life Technologies, Foster City, CA). Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds, followed by primer annealing and a final 78°C for 30 seconds. Molar concentrations of standards and sample DNA were converted into gene copies as shown in Obanda et al. [13].

### Statistical analyses

We used the MIXED procedure of SAS 9.4 for analysis of variance between treatments. Study I data were analyzed as a 2 × 2 factorial (OP and OR) and (LF and HF) in phase 1. Phase 2 data were analyzed as a 2 × 4 factorial (OP and OR) and (LF-LF, HF-HF, HF-LF, and HF-LFRS). For study II, total bacteria in fecal samples before and after knock down treatments were analyzed by paired t-tests in SAS by the PROC TTEST. Phase 2 data was analyzed as a 2 × 4 factorial (OP and OR) and (VM, NA, LX, WA). Main and interactive effects were considered significant at $P < 0.05$ and expressed as means ± SE. Further *a priori* comparisons used the alpha level divided by the number of comparisons.

## RESULTS

### Energy intake, body weight and total abdominal fat

In study I, as expected, OP rats had higher EBW and more abdominal fat accumulation than OR rats (P<0.001) (Figure 2 and Figure S2). The interaction between diet and phenotype was significant with OP rats fed a HF-HF diet gaining the most fat. The EBW and fat weight in OR rats was not different between the four dietary groups (P>0.05). In Study II, OP rats had more fat accumulation (P<0.0001) despite RS diet for all animals. The transplant had no effect on fat accumulation and EBW (P>0.05) (Figure 2; Figure S2). Overall, energy intake was lower in OR rats for phase 1 but was not diffrent in phase 2 of both studies (Figure S1).

### Fermentation levels and serum HOMA-IR and GLP-1

Fermentation variables for study I have been reported [13]. After study II, only phenotype effects were observed with greater amounts of SCFAs and low pH of cecal contents reflecting higher fermentation in OP rats. The antibiotics or Miralax treatments had no effects on fermentation levels compared to the water controls (Table 3).

Phenotype had no effect on HOMA-IR (P=0.89). The KD treatments similarly had no effect on HOMA-IR (P=0.76). Serum GLP-1 was higher among OP rats compared to OR rats but KD treatments had no significant effect (Table 3). The higher GLP-1 levels in OP rats is attributed to the more robust fermentation of RS compared to that in OR rats.

### Total bacteria in study II

The amounts of total bacteria for study I have been reported [13]. In study II, cecal contents after phase 1 were used in the transplant and hence were not available for analysis. Analysis of fecal samples after phase 1 when the rats were on a HF diet only, showed that OR rats had significantly higher amounts of total bacteria ($P<0.05$) (Figure 3A). Paired t-tests of total bacteria in fresh fecal samples collected before knock down treatment and 24 hours after treatment showed that the vancomycin + meropenem (VM) and the neomycin + ampicillin (NA) treatments both significantly lowered the bacteria loads compared to the water control ($P<0.005$). MiraLAX® had no significant effect with results not different from those of the water control (W) (Figure 3B). After phase 2 in which all rats had been switched to the HF-RS diet, OP rats had higher amounts of total bacteria ($P<0.02$; Figure 3C) and the methanogen M. smithii compared to OR rats (Figure S3).

### Amounts of *C. butyricum* in cecal contents

In study I, OR rats had higher levels *C.butyricum* in both phase 1 ($P<0.005$) and phase 2 ($P<0.001$) (Figure 4A–F); almost 6X more. The HF diet lowered *C. butyricum* levels ($P<0.005$) (Figure 4A–F). Study II similarly showed that OR rats had higher amounts of *C.butyricum*; more than 10X fold (Figure 4G–I). Dietary RS had no effect on the abundance of this species ($P=0.73$ and 0.86 in phase 1 and phase 2, respectively). In study II, the transplant had no effect on *C.butyricum* levels and the interaction of phenotype and transplant was not significant (Figure 4G–I). At the end of study II, *C. butyricum* levels in OR rats treated with the Neomycin and ampicillin (NA) antibiotic formulation before the transplant were significantly lower than in OR rats treated with Vancomycin and meropenem (VM), MiraLax and the water control (Fig 4H). The ratio of *C. butyricum* to total bacteria for both studies is shown in Figure 4 (J and K). Both studies revealed a higher proportion of *C. butyricum* compared to total bacteria in OR rats compared to OP rats. This difference was more pronounced in study II. We obseved a negative correlation between the abundance of *C. butyricum* and percent body fat; the correlation after study II had a Spearmans coefficient ($\rho$) of −0.63 (Figure 4L).

## DISCUSSION

We employed the outbred Sprague-Dawley CD rats in two independent studies to shed light on the association of the quantity of gut microbiota (assessed by abundance of the 16S rRNA gene) and *C. butyricum* in particular on the propensity to gain weight. Including both OR and OP rats enabled us to explore a possible link between the abundance of *C. butyricum* and propensity of the rats to develop obesity. We hypothesized that differences in gut microbiota contribute to the occurrence of the two obesity phenotypes presented by these rats. The abundance of *C. butyricum* was consistently higher in OR rats despite feeding *ad libitum*, equal energy intake by the end of the study and the microbiota transplant between the two phenotypes. OP rats responded to RS by robust fermentation as evidenced by significant increases in total bacteria, the archea *M. smithii* and SCFAs but, *C. butyricum* did not increase.

We focused on *C. butyricum* because it has been associated with pleiotropic positive effects in animal models. Specifically, it enhances butyrate production and reduces inflammation in the lower gut [7–10]. Furthermore, administration of *C. butyricum* as a probiotic enhances intestinal immune homeostasis and prevents diet induced weight gain [18–22]. While several other species have been associated with obesity prevention, and no one species in the gut functions in isolation, genus *Clostridium* has been linked to enhanced immune function by affecting the development and function of regulatory intestinal T cells (Treg cells). Treg cells in the intestinal mucosa maintain homeostasis and produce large amounts of anti-inflammatory cytokine IL-10 [21,22]. In addition to reducing body fat in response to a high fat diet, mice treated with *C. butyricum* CGMCC0313.1 exhibit reduced lipid accumulation in liver, improved serum lipid profiles, enhanced colon homeostasis, lower circulating insulin levels, improved glucose tolerance and insulin sensitivity [8, 18–22]. We used qPCR because in a previous study, analysis of bacterial composition by new generation sequencing as a screening tool for differences in relative abundance of operational taxonomic units did not detect *C. butyricum* [13].

We sought to determine if dietary manipulation can impact the proliferation of this bacterial species and whether a transplant can increase its proliferation in obesity prone rats. Obese resistant rats significantly had higher levels of *C. butyricum* irrespective of diet or antibiotic treament. While the ratio of *C. butyricum* to total bacteria was less than 0.006:1 in OP rats, it was 0.1:1 in OR rats (Figure 4F and G). The findings in both study I and study II corroborate a study by Shang et al. [7] who showed that treatment of mice with *C. butyricum* protected them against the development of high fat diet induced obesity. While the mechanism by which *C. butyricum* imparts its anti-obesity properties is not yet known, its mechanism of action as an anti-inflammatory agent has been confirmed to be through triggering the production of anti-inflammatory cytokine IL-10 by intestinal macrophages and promoting immune function and balance [10, 20–22]. The anti-obesity effects and anti-inflammatory effects may be interrelated. Thus abundance of *C. butyricum* levels in OR rats may have contributed to the more favorable metabolic profile as compared to OP rats. This bacterial species may play a role in metabolic processes such as, fat absorption, fat assimilation and/or storage and may contribute to maintenance of a lean body even in the presence of excess dietary calorie intake. The microbiota influences phenotype through direct contact with host cells or indirectly via the effect of bacterial metabolites on host cellular homeostasis and inflammatory mechanisms [1,2,9].

The OR microbiota was resilient and less susceptible to dietary changes. However, a HF diet negatively impacted *C. butyricum* levels. Dietary RS did not trigger robust butyrate production in OR rats which had abundant *C. butyricum*. This contrasts the work of Howarth and Wang [8] and Lopetuso et al. [9] who showed that *C. butyricum* selectively produces butyrate in LPS treated mice. Although RS changed the gut microbiota by increasing total bacteria and archaea in OP rats, *C. butyricum* levels did not change. It is likely that species that ferment RS increased as shown in our previous study [13]. The specific RS type used in this study may not be the ideal substrate for *C. butyricum*.

Consistent with our previous findings [13], total bacteria and the archaea *M. smithii* increased only in OP rats in response to robust RS fermentation. The ability of OR rats to remain lean

is thus not attributed to mechanisms involving robust fermentation of RS or an increase in production of butyrate.

Knock down of gut microbiota by antibiotics effectively created a niche for the transplant. However, the transplant did not promote a change in abundance of *C. butyricum* and did not transmit the parameters of the donor phenotype between OP and OR rats. Lowering the recipient bacterial load by antibiotics prior to the transplant did not increase establishment of the donor phenotypes. It has been reported that antibiotic pretreatment counterintuitively interferes with the establishment of an exogenous community [23] however, even pretreatment with MiraLAX which did not lower recipient load, did not result in an effective transplant. It is likely that species like *C. butyricum* are regulated by host factors; most likely the host's immune system. A review of fecal microbiota transplant studies shows successful results in healing the blooming of *C. difficile* [23,25]. One significant observation is that while total bacteria levels were restored in both OP and OR rats 4 weeks after administering antibiotics, *C. butyricum* levels remained lower and were not restored to pre-antibiotic levels in rats given neomycin and ampicillin (NA) (Figure 2H). This suggests that antibiotic intake may negatively affect levels of beneficial bacteria for long term periods and/or result in growth of alternative species after the antibiotic regime.

In summary, the microbiota changes due to dietary interventions occurred mainly in OP rats. The microbiota of OR rats was more resilient to dietary changes and had consistently higher abundance of *C. butyricum* despite less fermentation of RS. This bacterial species may impact nutrient absorption and assimilation mechanisms in ways that are not yet known.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## FUNDING

Research funded by LSU AgCenter (NIFA 94268) and Ingredion Incorporated. Starches were gifts from Ingredion Incorporated. Research partially supported by NIH NORC Center Grant #P30DK072476 entitled "Nutritional Programming: Environmental and Molecular Interactions."

## REFERENCES

[1]. Ley RE, Backhed F, Turnbaugh PJ, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc. Natl. Acad. Sci. USA 2005, 102, 11070–5. [PubMed: 16033867]

[2]. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity Nature. 2006, 444, 1022–3. [PubMed: 17183309]

[3]. Madsen AN, Hansen G, Paulsen SJ, Lykkegaard K, Tang-Christensen M, Hansen HS. Long term characterization of the diet induced obese and diet resistant rat model: a polygenetic rat model mimicking the human obesity syndrome J. Endoc 2010, 206, 287–6.

[4]. Levin BE, Dunn-Meynell AA, Balkan B, Keesey RE. Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats. Am J Physiol. 1997, 273(2Pt2): R725–30. [PubMed: 9277561]

[5]. Adam CL, Williams PA, Dalby MJ, Garden K, Thompson LM, Richardson AJ, Gratz SW, Ross AW. Different types of soluble fermentable dietary fibre decrease food intake, body weight gain and adiposity in young adult male rats. Nutr Metab (Lond) 2014, 11, 36. [PubMed: 25152765]

[6]. Schroeder N, Marquart LF, Gallaher DD. The role of viscosity and fermentability of dietary fibers on satiety- and adiposity-related hormones in rats. Nutrients 2013, 5, 2093–2113. [PubMed: 23749206]

[7]. Shang H, Sun J, Chen YQ. *Clostridium Butyricum* GMCC0313.1 modulates lipid profile, insulin resistance and colon homeostasis in Obese Mice. PLOS One. 2016, 11,4, e0154373. DOI: 10.1371/journal.pone.0154373.eCollection2016. [PubMed: 27123997]

[8]. Howarth GS, Wang H. Role of Endogenous Microbiota, Probiotics and their Biological Products in Human Health. Nutrients. 2013, 5,1,58–81. [PubMed: 23306189]

[9]. Lopetuso LR, Scaldaferri F, Petito V, Gasbarrini A. Commensal Clostridia: leading players in the maintenance of gut homeostasis. Gut Pathogens. 2013, 5, 23. [PubMed: 23941657]

[10]. Hayashi A, Sato T, Kamada N, Mikami Y et al. A single Strain of Clostridium butyricum Induces Intestinal IL-10-producing macrophages to suppress acute experimental colitis in mice. Cell host and Microbe. 2013. 13(6), 711–722. [PubMed: 23768495]

[11]. Ellekilde M, Selfjord E, Larsen CS, Jakesevic M, Rune I, Tranberg, B, Vogensen FK, Neilsen DS, Licht MI, Hansen AK, Hansen CH. Transfer of gut microbiota from lean and obese mice to antibiotic-treated mice. Scientific Reports. 2014, 4, 5922. [PubMed: 25082483]

[12]. Jalanka J, Salonen A, Salojärvi J, Ritari, Immonen, O, Marciani L, Gowland P, Hoad C, Garsed K, Lam C, Palva A, Spiller RC, de Vos VM. Effects of bowel cleansing on the intestinal microbiota. Gut. 2014, 64,1562–8. [PubMed: 25527456]

[13]. Obanda DN, Page R, Guice J, Raggio AM, Husseneder C, Marx B, Stout RW, Welsh DA, Taylor CM, Luo M, Blanchard EE, Bendicks Z, Coulon D, Keenen M. Obesity. CD Obesity-Prone rats, but not obesity-resistant rats, robustly ferment resistant starch without increased weight or fat accretion. Obesity. 2018, 26,3, 570–7. [PubMed: 29464911]

[14]. Goldsmith F, Guice J, Page R, Welsh DA, et al. Obese ZDF rats fermented resistant starch with effects on gut microbiota but no reduction in abdominal fat. Mol Nutr Food Res 2017;61.

[15]. Belenguer A, Duncan SH, Calder AG, Holtrop G, Louis P, Lobley GE, Flint HJ. Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. Appl. Environ. Microbiol 2006. 72, 5, 3593–9. [PubMed: 16672507]

[16]. Bartosch S, Fite A, Macfarlane GT, McMurdo ME. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using Real-Time PCR and effects of antibiotic treatment on the fecal microbiota. Appl. Environ. Microbiol 2004, 70, 6, 3575–81. DOI: 10.1128/AEM.70.6.3575-3581.2004 [PubMed: 15184159]

[17]. Dridi B, Henry M, El Khéchine A, Raoult D, Drancourt M. High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. PLOS one. 2009, 4, e7063. [PubMed: 19759898]

[18]. Jia L, Shan K, Pan LL, Feng N, Lv Z, Sun Y, Wu C, Zhang H, Chen W, Diana J, Sun J, Chen YQ. *Clostridium butyricum* CGMCC0313.1 protects against autoimmune diabetes by modulating intestinal immune homeostasis and inducing pancreatic regulatory T Cells. Front. Immunol 2017, 8, 1345 [PubMed: 29097999]

[19]. Gao Q, Qi L, Wu T, Xia T, Wang J. Immunomodulatory effects of Clostridium butyricum on human enterocyte-like HT-29 cells. Animal cells and Systems. 2013. 17 (2): 21–126.

[20]. Yang CM, Cao GT, Ferket PR, Liu TT. et al. Effects of probiotic, *Clostridium butyricum*, on growth performance, immune function, and cecal microflora in broiler chickens. Poult Sci. 2012, 91 (9, 2121–2129. [PubMed: 22912445]

[21]. Nagano Y, Itoh K, Honda, K. The induction of Treg cells by gut-indigenous Clostridium. Curr Opin Immunol. 2012. 24(4):392–7. DOI: 10.1016/j.coi.2012.05.007 [PubMed: 22673877]

[22]. Petersen C, Bell R, Klag KA, Kendra A et al. T cell–mediated regulation of the microbiota protects against obesity. Science. 2019. 365: eaat 6451.

[23]. Manichanh C, Reeder J, Gibert P et al. Reshaping the gut microbiome with bacterial transplantation and antibiotic intake. Genome Research. 2010; 20(10):1411–1419. [PubMed: 20736229]

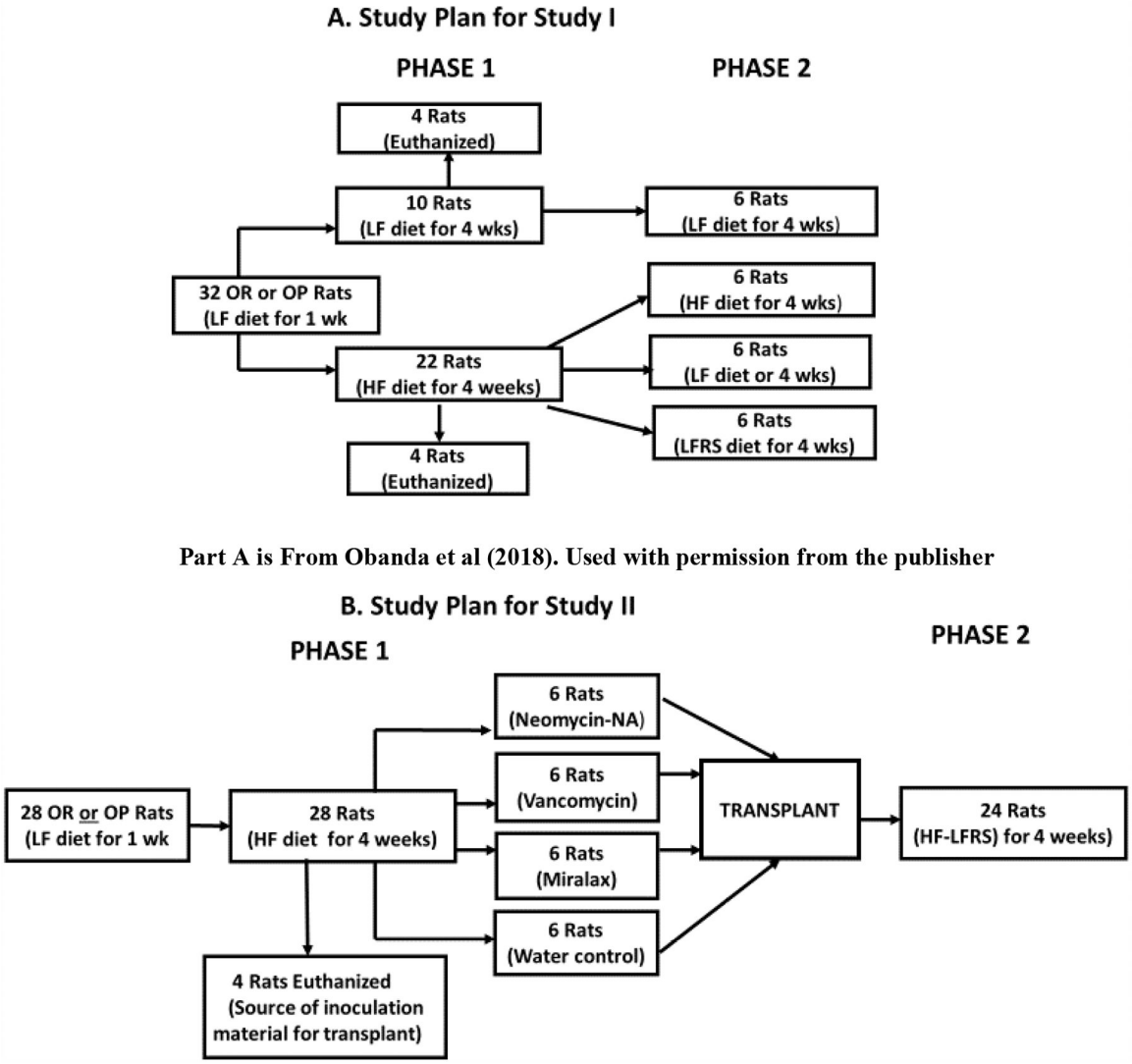
[24]. Youngster I, Sauk J, Pindar C, Wilson RG, Kaplan JL, Smith MB, Alm EJ, Gevers D, Russell GH, Hohmann EL. Fecal microbiota transplant for relapsing *Clostridium difficile* infection using

a frozen inoculum from unrelated donors: a randomized, open-label, controlled pilot study. Clin. Infect. Dis 2014, 58, 11, 1515–22. [PubMed: 24762631]

[25]. Satokari R, Mattila E, Kainulainen V, Arkkila PE. Simple faecal preparation and efficacy of frozen inoculum in faecal microbiota transplantation for recurrent *Clostridium difficile* infection--an observational cohort study. Aliment. Pharmacol. Ther, 2015, 41,1, 46–53. [PubMed: 25355279]

## Highlights

- Rats that are obese resistant have 6–10X more *C. butyricum* in their cecal contents compared to rats that are obese prone. Prospensity to accumulate body fat negatively correlates with *C. butyricum* levels.
- Dietary resistant starch does not increase the proliferation of *C. butyricum*.
- A high fat diet reduces amounts of *C. butyricum*.
- The ability of obese resistant rats to remain lean is not attributed to mechanisms involving robust fermentation of RS or an increase in production of butyrate.
- Four weeks after an antibiotic regime (neomycin and ampicillin) total bacteria levels are restored but levels of *C. butyricum* are not restored.




**Part A is From Obanda et al (2018). Used with permission from the publisher**

**Figure 1. Work flow and study plan for studies I and II**

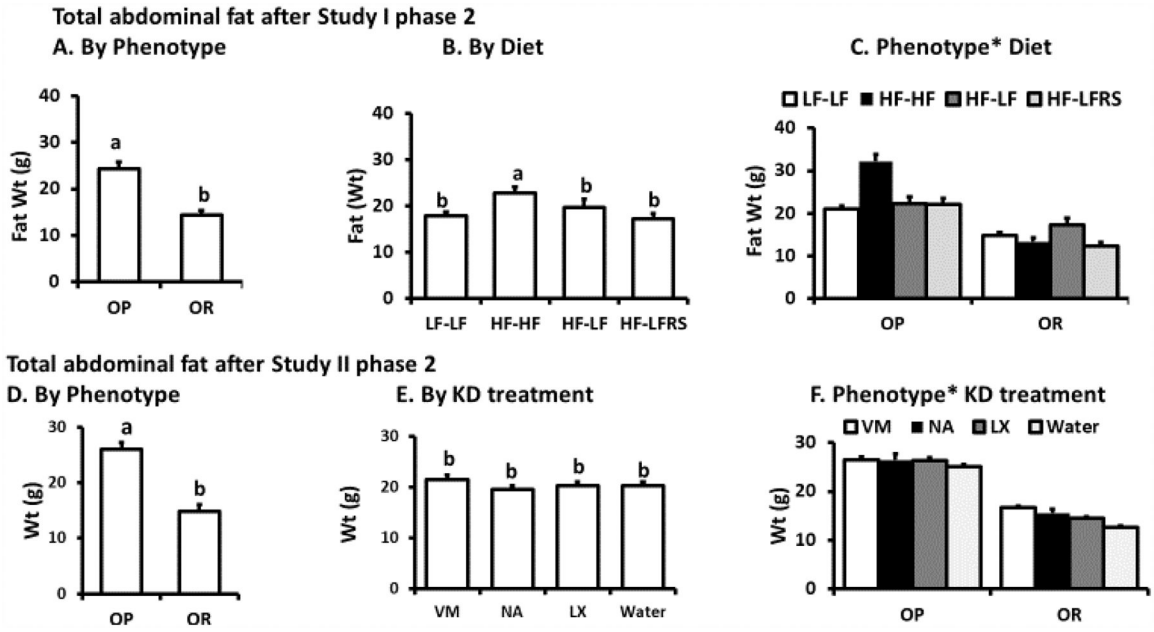



**Figure 2. Total abdominal fat was higher in OP rats irrespective of diet type, antibiotic treatment or the transplant.**

The sum of the weights of the retroperitoneal, perirenal and epididymal fat pads were determined at euthanasia. (**A**) The letters denote significant difference ($P<0.0019$) between OP and OR rats. (**B**) The letters denote significant difference between the HF-HF diet and the other diets ($P<0.046$). **(C)** The interaction between diet and phenotype was significant ($P<0.05$). **(D)** The letters denote a significant difference ($P<0.0019$) between OP and OR rats. **(E)** The Knock down-transplant treatment had no effect on fat gain. **(F)**. The interaction between phenotype and knock down-transplant was not significant.

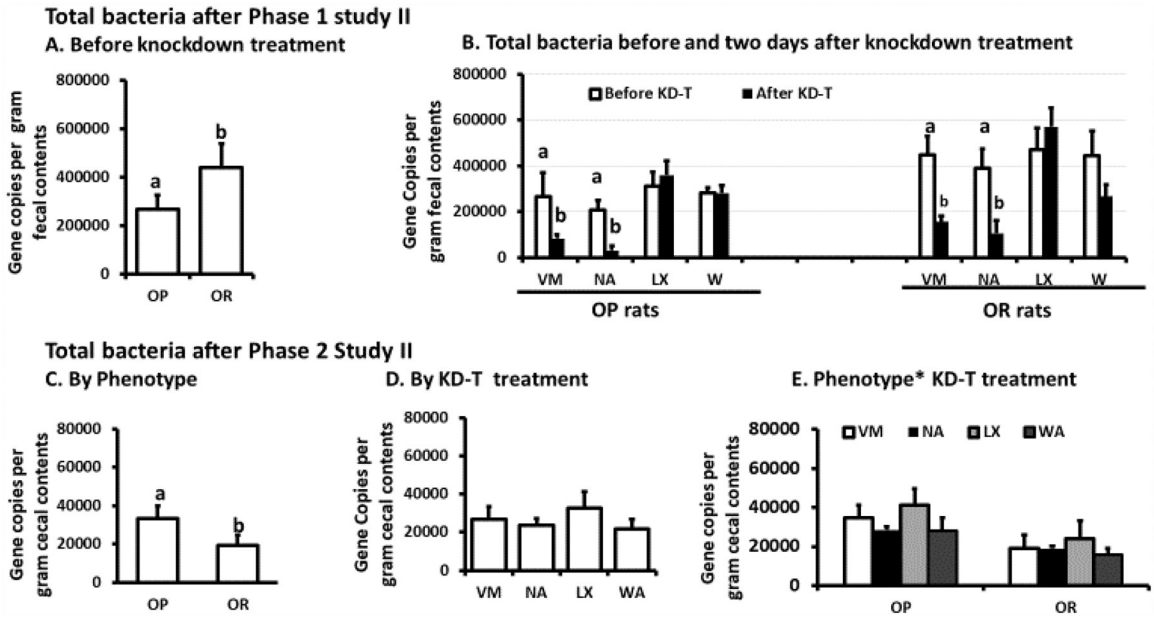



**Figure 3. Total bacteria in study II**

**(A)** qPCR was conducted on DNA extracted from fresh fecal samples collected before knock down treatments. The letters denote significant differences between the OR and OP rats P<0.005. (**B**) The letters denote a significant difference before and after knock down treatments (P<0.0165). **(C)** DNA was extracted from cecal samples after euthanasia. The letters denote significant differences between the OR and OP rats P<0.005 after switching to HFRS diet. (**D**) No significant differences were observed among knock down-transplant treatments. (**E**) The interaction between phenotype and knock down-transplant treatmnet was not signficant.

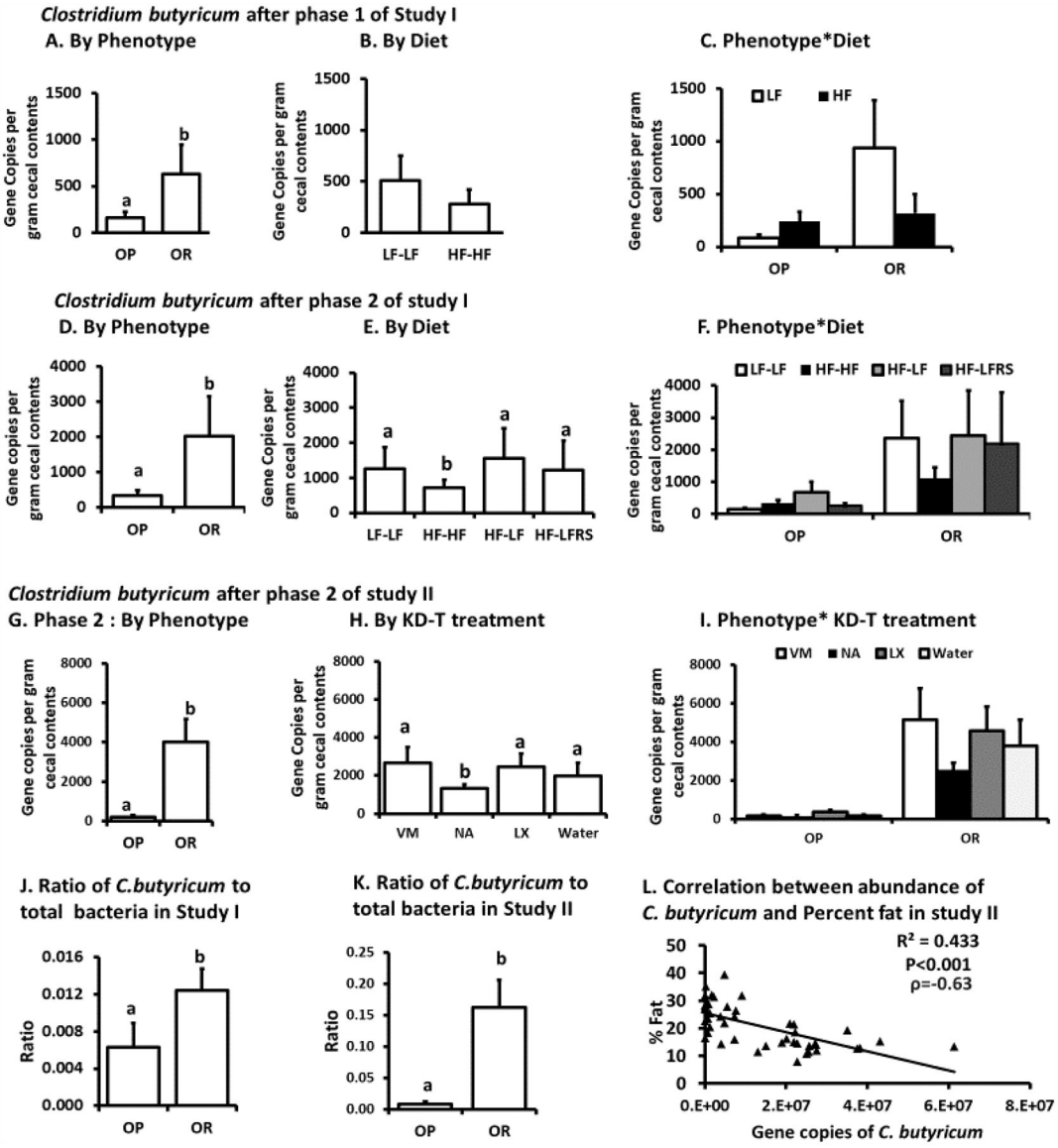



**Figure 4. Abundance of C. butyricum was higher in OR rats and correlates with percent body fat.**

*C. butyricum* levels in cecal DNA were quantified by qPCR. (**A**) The letters denote a significant diffrence between OP and OR phenotypes (P<0.012). (**B**). Diet had no significant effect in phase 1 and (**C**) the interaction between diet and phenotype was not significant. In phase 2, (**D**) The letters denote a significant difference between OP and OR phenotypes at (P<0.005). (**E**) The letters denote significant differences between the HF-HF diet and the other three diets (P<0.05) and (**F**) the interaction of diet and phenotype was not significant. (**G**)The letters denote a significant difference between OP and OR rats (P <0.0001). (**H**) NA antibiotics resulted in lower C. butyricum levels (P <0.05). (**I**) The interaction between phenotype and knock down-transplant treatment was not signficant. **(J)** The letters denote a significant difference between OP and OR phenotypes (P<0.005). **(K)** The letters denote

a significant difference between OP and OR rats (P<0.005). **(L)** A negative correlation between *C. butyricum* levels and body fat was observed.

**Table 1.**

Phenotype, diet, and Knock down treatment acronyms for phases 1 and 2 of both studies

| |
|---|
| OP:Obesity-prone |
| OR: Obesity-resistant |
| LF:Low fat diet |
| HF:High fat diet |
| LF-LF: Low-fat diet in phase 1 and phase 2. |
| HF-HF: High-fat diet in phase1 and phase 2. |
| HF-LF: High-fat diet in phase 1 with a switch to a low-fat diet for phase 2 |
| HF-LFRS: High-fat diet in phase 1 with a switch to a low-fat diet containing 20% resistant starch in phase 2 |
| HF-HFRS: High-fat diet in phase 1 with a switch to a high-fat diet containing 20% resistant starch in phase 2<br>NA:Neomycin and ampicillin as knock down treatment. |
| VM:Vancomycin and meropenem as knock down treatment. |
| LX:Miralax® as knock down treatment. |
| WA:Water control as knock down treatment and transplant with water as a control. |

**Table 2.**

Diets for obesity prone and obesity resistant rats

| **Diet components** | | **LF Diet** | | **HF Diet** | |
|---|---|---|---|---|---|
| | | **LF (g)** | **LF-RS (g)** | **HF (g)** | **HF-RS (g)** |
| **Ingredients (g)** | ***Energy value (Kcal/g)*** | | | | |
| [a]AMIOCA™ starch | 3.5 | 521.1 | 147.1 | 405.7 | 31.8 |
| [b]HI-MAIZE® 260 RS | 2.8 | 0 | 472.4 | 0 | 472.4 |
| Sucrose | 4.0 | 100 | 100 | 100 | 100 |
| Casein | 3.50 | 140 | 140 | 140 | 140 |
| Cellulose | 0 | 150.8 | 52.4 | 106.2 | 7.7 |
| Corn oil | 8.84 | 40 | 40 | 100 | 100 |
| Lard | 9.00 | 0 | 0 | 100 | 100 |
| Mineral mix (AIN-93M) | 0.88 | 35 | 35 | 35 | 35 |
| Vitamin mix (AIN 93) | 3.87 | 10 | 10 | 10 | 10 |
| Choline chloride | 0 | 1.3 | 1.3 | 1.3 | 1.3 |
| L-Cystine | 4 | 1.8 | 1.8 | 1.8 | 1.8 |
| **Total weight (Kcal)** | | **1000g** | **1000** | **1000** | **1000** |
| | | **(3160.2)** | **(3160.1)** | **(4182.5)** | **(4182.7)** |

[a]AMIOCA™ is a 100% amylopectin cornstarch product from Ingredion (Bridgewater, NJ).

[2]HI-MAIZE® 260 is high-amylose cornstarch from Ingredion Incorporated (Bridgewater, NJ).

The batch used was 42.3% resistant starch based on wet weight for use in diet.

**Table 3.**

Fermentation variables: pH, SCFAs in cecal contents and serum GLP-1 and HOMA-IR after Phase 2 of study II

| SCFA/pH | Treatment group | OP | OR | | P value (Phenotype) | P value (Knockdown) | Pooled SEM |
|---|---|---|---|---|---|---|---|
| Acetate (Mmol/cecum) | VM | 0.448 * | 0.074 | | <0.001 | 0.6925 | 0.011 |
| | NA | 0.536 * | 0.122 | | | | |
| | LX | 0.510 * | 0.094 | | | | |
| | Water | 0.490 * | 0.118 | | | | |
| Propionate (Mmol/cecum) | VM | 0.037 * | 0.006 | | <0.001 | 0.7109 | 0.004 |
| | NA | 0.036 * | 0.002 | | | | |
| | LX | 0.031 * | 0.004 | | | | |
| | Water | 0.043 * | 0.011 | | | | |
| Butyrate (Mmol/cecum) | VM | 0.101 * | 0.018 | | <0.005 | 0.4526 | 0.011 |
| | NA | 0.115 * | 0.025 | | | | |
| | LX | 0.107 * | 0.023 | | | | |
| | Water | 0.076 * | 0.014 | | | | |
| pH | VM | 5.64 * | 6.9 | | 0.005 | 0.453 | 0.12 |
| | NA | 5.78 * | 7.2 | | | | |
| | LX | 5.65 * | 6.8 | | | | |
| | Water | 5.89 * | 6.9 | | | | |
| Serum GLP-1 (Pmol/L) | VM | 1.22 * | 1.02 | | <0.05 | 0.09 | 0.145 |
| | NA | 1.32 * | 0.98 | | | | |
| | LX | 1.82 * | 1.18 | | | | |
| | Water | 1.48 * | 1.21 | | | | |
| HOMA-IR | VM | 1.53 | 2.11 | | NS | NS | 0.23 |
| | NA | 2.01 | 1.51 | | | | |
| | LX | 1.47 | 1.81 | | | | |
| | Water | 2.02 | 1.51 | | | | |

* P value denotes significant differences between OP and OR phenotypes at P<0.05. No differences were observed between knockdown treatment types.