THE UNIVERSITY OF CHICAGO MEDICINE
AT THE FOREFRONT OF MEDICINE

imc FH KREMS
UNIVERSITY OF APPLIED SCIENCES / AUSTRIA

The role of *Clostridium bifermentans* in small intestinal lipid absorption using *in vitro* and *in vivo* models

AUSTRIAN
MARSHALL PLAN FOUNDATION
VIENNA | AUSTRIA

Final Report

by

Saskia URLASS

submitted at the

Austrian Marshall Plan Foundation

Bachelor programme: Medical and Pharmaceutical Biotechnology

Internal Supervisor: Barbara Entler, Prof.(FH) Dr.

External Supervisor: Kristina Martinez, PhD RD

Statutory Declaration

"I declare in lieu of an oath that I have written this bachelor paper myself and that I have not used any sources or resources other than stated for its preparation. I further declare that I have clearly indicated all direct and indirect quotations. This bachelor paper has not been submitted elsewhere for examination purposes."

Datum: 30.03.2017

Saskia Urlaß

Acknowledgment

First of all, I would like to express my gratitude to my external supervisor Kristina Martinez, PhD, RD for being such a great support throughout my internship and giving me the opportunity of being part of such an exciting project. I really appreciate her patience, enthusiasm, and knowledge sharing. Moreover, I am also grateful for her help after my time in the States.

I would also like to thank the principal investigator Dr. Eugene B. Chang for giving me the opportunity to work in his laboratory, and thereby giving me the possibility to gain valuable work experience in research and learning from incredible people. Additionally, I am grateful to all laboratory members, who warmly welcomed me into their team and special thanks to the administrator of the laboratory, Francine Vukovich for organizing my stay, including the visa and therefore making it possible for me to actually have this amazing overseas experience.

I am especially thankful to the Austrian Marshall Plan Foundation for the financial support, which enabled myself to attend the internship at the University of Chicago.

I would like to give my sincere thanks to my internal supervisor Dr. Barbara Entler for her great supervision and great know-how.

Abstract

The worldwide obesity epidemic has tripled in the last decade and is still rising at an alarming pace, thereby afflicting millions of people with associated metabolic disorders, including type 2 diabetes (T2D) and cardiovascular diseases. (WHO, 2016) The gut microbiota has been implicated in playing a role in the development of obesity. The gastrointestinal tract (GIT) harbours 100 trillion cells of 400 species. Since the breakthrough of next-generation sequencing technology, the gut microbiota research made enormous progress, due to the identification of previously unknown microorganisms (MOs). (Kasubuchi et al., 2015) In addition, germ-free (GF) animal studies revealed new insights regarding the functional impact of microbes on host nutrient processing and metabolism. For example, protection against diet-induced obesity (DIO), due in part to malabsorption of fat, has been demonstrated in GF mice. (Bäckhed et al., 2007)

Preliminary data from Eugene Chang's laboratory showed that mice fed a high-fat diet have increased relative abundance of Clostridiaceae in the small intestine, determined by 16S rRNA sequencing. Additionally, operational taxonomic units that were significantly elevated in high-fat (HF) vs low-fat (LF) conditions shared 96% sequence similarity to *Clostridium bifermentans*. Therefore, my studies focused on whether or not *C. bifermentans* induces lipid absorption using *in vitro* and *in vivo* models.

Herein, we demonstrate that conditioned media (CM) from *C. bifermentans* induced the expression of canonical re-esterification enzymes important for lipid absorption in small intestinal organoid cultures. *C. bifermentans* supplementation *in vivo* significantly increased the expression of re-esterification enzyme Dgat2 that is especially important in triacylglycerol synthesis, and thereby contribute to increased lipid absorption and transport. Taken together, our findings suggest that small intestinal microbes (i.e. *C. bifermentans*) are key players in lipid absorption, and that these host-microbe interactions may ultimately contribute to obesity and associated metabolic disorders.

Table of Content

Statutory Declaration....................II
Acknowledgment....................II
Abstract....................III
Table of Content.................... IV
List of Figures and Illustrations.................... VI
List of Abbreviations.................... VII

1 Introduction....................1
1.1 Obesity and gut microbiota....................1
1.1.1 Composition of the gut microbiota....................1
1.1.2 Dietary impact on gut microbiota....................4
1.2 Gut microbes and Lipid absorption....................6
1.2.1 Small intestinal Lipid absorption....................6
1.2.2 Gut microbiota promotes lipid absorption....................8
1.3 Clostridiaceae....................10
1.3.1 Obesogenic effect of *Clostridium ramosum*....................10
1.3.2 *Clostridium bifermentans*....................12
1.4 Specific Aims....................12
1.4.1 Aim 1....................12
1.4.2 Aim 2....................13
2 Materials and Methods....................14
2.1 Generation and Culturing of Enteroids....................14
2.1.1 Harvesting enteroids....................14
2.1.2 Passaging enteroids....................15
2.2 Treating generated Enteroids with conditioned media (CM)....................15
2.2.1 Preparation of RCM....................15
2.2.2 Preparation of CM....................16
2.2.3 Treating enteroids with conditioned media....................16
2.3 Generation and culturing of Enteroid Monolayers....................20
2.3.1 Fatty Acid Uptake Assay....................20
2.4 Animal Study....................22
2.4.1 Experimental Design....................22
2.4.2 Establishing growth curve for *C.bifermentans*....................23

2.4.3 DNA extraction 25
2.4.4 Tissue collection 26
3 Results & Discussion 28
3.1 Conditioned media from *C. bifermentans* increases expression of genes involved in lipid absorption 28
3.2 Enteroid Monolayer Fatty Acid Uptake Assay 29
3.3 Animal Study 31
3.3.1 Body weight & Food intake 31
3.3.2 *In vivo* supplementation with *C. bifermentans* increases expression of genes involved in lipid absorption 32
4 Conclusion and Future outlook 34
List of References 36

List of Figures and Illustrations

Figure 1 Illustrates the main factors affecting microbial composition. 2
Figure 2 Illustrates the microbial development during life.. 3
Figure 3 Illustrates the total increase in body weight measured by DEXA 5
Figure 4 Illustrates lipid absorption in the small intestine 6
Figure 5 Illustrates Triacylglycerol synthesis pathway...7
Figure 6 Illustrates increased feacal lipid excretion of GF mice on HF diet. 8
Figure 7 GF mice on HF diet are resistant to DIO and diet-induced Insulin sensitivity compared to their CONVD counterparts 9
Figure 8 Illustrates relative abundance of Clostridiaceae......................................10
Figure 9 Illustrates obesogenic effect of *C.ramosum* 11
Figure 10 Illustrates the impact of C.ramosum on liver metabolism 11
Figure 11 Illustrates the experimental design of the Animal Study........................23
Figure 12 Illustrates growth curve for *C. bifermentans*..29
Figure 13 Illustrates increased gene expression of re-esterification enzymes 29
Figure 14 Illustrates 3H-Oleic acid uptake in duodenal organoid cultures.............30
Figure 15 Illustrates Body weight and Food intake of mice supplemented with *C. bifermentans and control*..31
Figure 16 Illustrates gene expression of pparγ, dgat2, fabp2 and cd36 measured in duodenal mucosal scrapings via qPCR..32

List of Abbreviations

C.bif	*Clostridium bifermentans*
C.ram	*Clostridium ramosum*
cck	Cholecystokinin
CD36	Fatty acid translocase
CM	Conditioned media
CONVD	Conventionalised
CPM	Counts per minute
DEXA	Dual-energy x-ray absorptiometry
DGAT	Diacylglycerol
Dgat2	Diacylglycerol O-Acyltransferase 2
DIO	Diet-induced obesity
FA	Fatty acid
FABP2	Fatty acid binding protein 2
FFA	Free fatty acid
FMT	Faecal microbiota transplant
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GF	Germ-free
GIT	Gastrointestinal tract
Glut 2	Glucose transporter 2
HF	High-fat
LF	Low-fat
Lgg	*Lactobacillus rhamosus gg*
MAG	Monoacylglycerol
MF	Milk fat
Mogat2	Monoacylglycerol O-Acyltransferase 2
MOs	Microorganisms
PPAR-γ	Peroxisome-proliferator-activated receptor gamma
PUFA	polyunsaturated fat
RCM	Reinforced Clostridial Media

sct	Secretin
SIHUMI	Simplified human intestinal microbiota
SPF	Specific Pathogen Free
T2D	Type 2 Diabetes
TAG	Triacylglycerol

1 Introduction

1.1 Obesity and gut microbiota

In the last decade the worldwide obesity epidemic has tripled and therefore constitutes to a rising health issue in westernized countries. It is very alarming how this epidemic leads to metabolic disorders, including T2D, cardiovascular diseases and specific cancer types. Many factors contribute to obesity including, increased food intake, lack of physical activity, genetics, sleep disruption and as recent studies revealed, the gut microbiota is an important key player in the development of obesity. (Hartstra et al., 2015)

1.1.1 Composition of the gut microbiota

The bacterial population that resides in the GIT, includes over 100 trillion cells of 400 species and is collectively called the gut microbiota. In addition to bacterial cells, yeast, archea and viruses inhabit the GIT. As most of the gut microbes are anaerobic, it is rather difficult to analyse their identity and functionality, although methods like 16S rRNA sequencing (method to identify and compare microbes in a sample) or metagenomics (genomic analysis of microbial DNA) are now available (Kasubuchi et al., 2015). Since the breakthrough of next-generation sequencing technology, the gut microbiota research made enormous progress through identifying different bacterial taxonomic units in a relative short time frame. (Reuter et al., 2015) Additionally, use of GF animals revealed new insights of microbe-mediated communication that drive obesity. For example, GF mice that lack microbiota are resistant to diet-induced obesity. (Rabot et al., 2010)

Bacterial community harboured by the GIT continuously increases from the stomach towards the large intestine, starting with 10^1 to 10^3 bacteria/gram of bacterial cells in the stomach and duodenum, 10^4 to 10^7 bacteria/gram in the jejunum and ileum and 10^{11} to 10^{12} bacteria/gram in the colon. (Baothman et al., 2016) Despite fewer numbers, a variety of microbes reside throughout the small intestine within its three

regions: duodenum, jejunum and ileum. The families that dominate this region include Enterobacteiaceae and Lactobacillaceae. The dominating families of the colon are Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae and Ruminococcaceae. The wall of the colon is composed out of folds creating an interfold region, which harbours Rumonococcaceae and Lachnospiriaceae (Donaldson et al. 2015).

The composition of the gut microbiota continuously changes through life, which is caused by different factors such as diet, host genetics and drug intake especially antibiotics in early life, see *Figure 1*. Also cultural and culinary diversity play an important role in affecting the microbiota (Baothman et al., 2016).

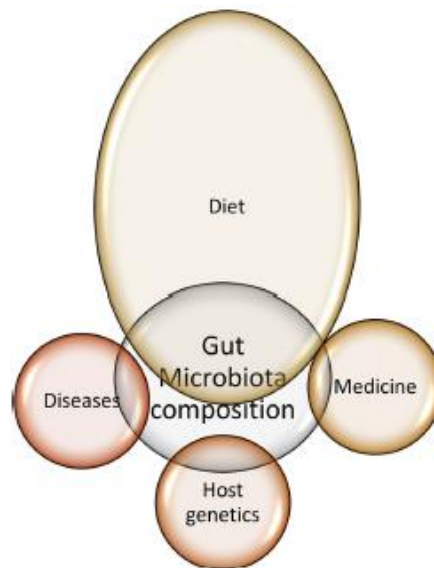

Figure 1 The main factors affecting microbial composition, highlighting diet as the main contributor. Not demonstrated the geographic diversity including regional differences, such as hygiene. (Baothman et al., 2016).

Not only does the human gut microbiota differ in different parts of the GIT, but also during life stages, including 1) Birth to infancy, 2) Adolescence and 3) Old age. Notably, each stage is associated with changes in dietary intake (Sekirov et al., 2010).

During pregnancy, the GIT of the infant is free of microbes until normal birth, when it is exposed to vaginal microbes of the mother. If the infant is born through caesarean section, the infant is exposed to different bacteria, which may result in a weaker immune system. After three days, the microbiota of the infant that was delivered naturally has a more versatile abundance of Bifidobacterium spp. than the infant delivered by caesarean section. Feeding methods also influence the infant microbiota, as breast fed babies have a different microbiota than formula fed babies. Intriguingly, formula fed babies are more likely to develop allergies and eczema (Martin et al. 2010). When babies are introduced to solid foods their gut microbiota shifts again to an even more diverse microbiota, but afterwards it remains relatively unchanged until reaching an older age, see *Figure 2*. Also during an older age the gut microbiota changes continuously, as the gut microbiota is influenced by many factors, such as host genetics and drugs (antibiotics) but mainly by diet. The gut microbiota responds immediately to dietary changes, which has been a major focus of the microbiota field. (David et al., 2014)

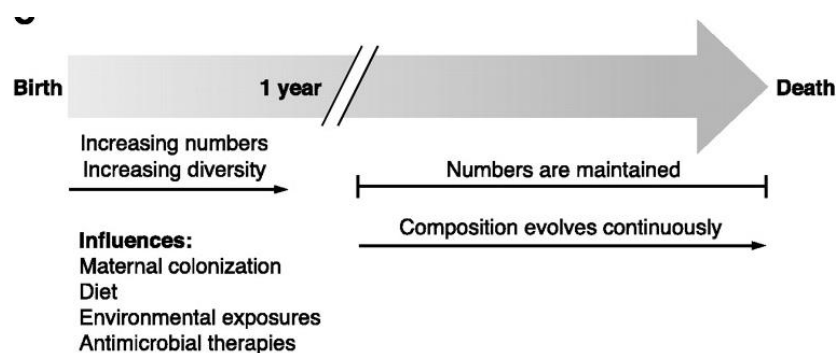

Figure 2 Microbial development during life. In the first year most fundamental changes occur with increasing diversity and bacterial community in the infants GIT. During adulthood the numbers of bacteria are relatively maintained, but can be affected by different factors such as diet (Sekirov et al., 2010).

1.1.2 Dietary impact on gut microbiota

“We are what we eat” and so is our microbiota. Not surprisingly diet is considered the main effector from birth on in altering the gut microbiota. (Carmody et al., 2015) Breastfeeding is a crucial factor in the development of the infant microbial composition and for the development of an immune system that protects the infants of infections and obesity later during their life (Robinson, 2012). Breast milk contains immunologically active compounds such as antibodies fighting against pathogens. Additionally, breast milk comprises complex polysaccharides that favour the growth of certain microorganisms (MOs), which may contribute to the infant healthy state, as it maintains the profiting microbial composition (Ballard, 2013).

De Filippo et. al compared faecal microbiota from children from a rural African village (Burkina Faso, Africa), where the population is on a fiber-rich diet and from Europe (Florence, Italy), where the diet is rather modernly westernized (high in protein and fibre lacking). With 16S rRNA sequencing and biochemical analysis, clear differences between those groups were shown. The children from Burkina Faso had a significant increase in Bacteroidetes and decrease in Firmicutes with a bacterial abundance of Prevotella and Xylanibacter, which are characteristic for cellulose/xylan hydrolysis. European children were lacking those. Additionally more short-chain fatty acids appeared in the children from Burkina Faso compared to the European children. It is presumed that the diet rich in fibre serves as a protection from inflammation, as the children living in developing countries are low on vaccines and any kind of drugs. Whereas in Europe due to improved sanitation, use of antibiotics and availability of vaccines, the European children are more prone to allergies and autoimmune disorders. (De Filippo et al., 2010).

The dietary impact on microbiota has been also demonstrated in several animal studies. For example, in Bäckhed et al. studies it was revealed that GF mice are resistant to DIO, when fed a HF diet (Bäckhed et al., 2007). In another study by Turnbaugh et al. humanized mice were created by fecal microbial transplants (FMT) from human subjects (donors were either on HF or LF diet) into a gnotobiotic mouse model (C57BL/6J) for two weeks, which were fed a LF diet rich in Polysaccharides. By metagenomic analysis it was shown that switching conventionalized (CONVD)

mice to a HF diet, their microbial community changed within one day and led to an obese phenotype. Under LF diet there was no significant difference, see *Figure 3* (Turnbaugh et al., 2009), suggesting that FMT could have therapeutic potential for metabolic diseases.

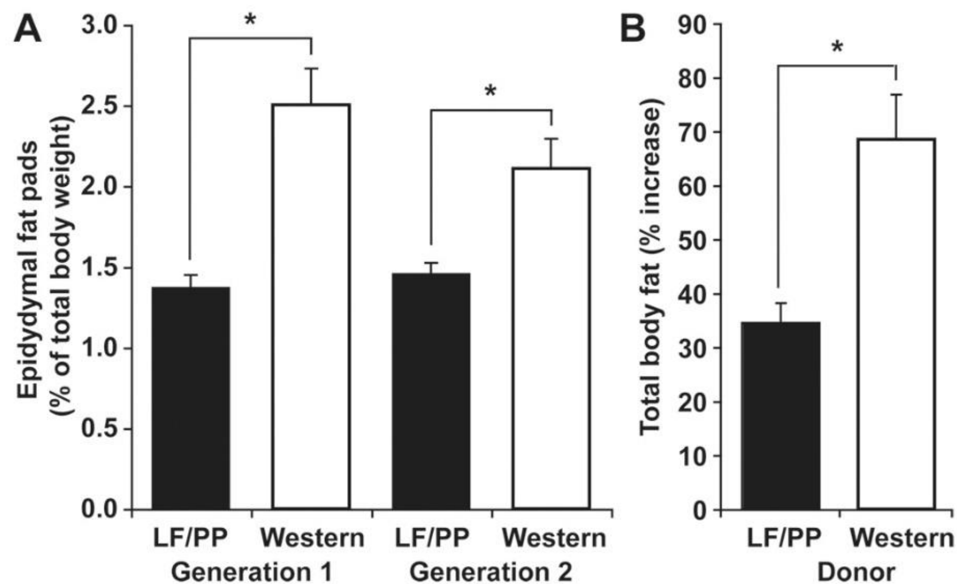

Figure 3 (A) Shows percentage of Epidymal fat pads in correlation to the total body weight of CONVD mice with human faeces, which were either on LF or HF diet (B) Shows the total increase in body weight measured by DEXA (dual-energy x-ray absorptiometry), which is technique used to produce detailed images of the body components (Turnbaugh et al., 2009).

1.2 Gut microbes and Lipid absorption

Microbial-mediated regulation of lipid absorption and digestion may contribute to obesity and associated metabolic disorders. While a connection has been demonstrated between microbes and lipid absorption the mechanisms behind this host-microbe interaction remain unknown. (Martinez et al., 2016)

1.2.1 Small intestinal Lipid absorption

Dietary lipids occur in the form of Triacylglycerol (TAG) that are composed out of a glycerol backbone, where each carbon is linked to a free fatty acid (FFA). Once dietary fat reaches the small intestine, it is emulsified by bile to form micelles, which increases the ability of lipases to digest TAG into FFA and monoacylglycerides (MAG). Entry of fat into the small intestine triggers the release of enteroendocrine hormones that signal the secretion of pancreatic lipase (cleaves ester bonds of TAG), which degrades TAG into FFA and MAG. Once FFA and MAG are transported into the enterocyte the re-synthesis of FFAs and MAG into TAG takes place, which are further packaged into chylomicrons. Chylomicrons are lipoprotein transport particles derived from dietary fat, which enter the lymph vessels and eventually dumped into the systemic circulation via the thoracic duct. FFA cleaved from chylomicrons are then available for oxidation or storage in adipose tissue and liver, see *Figure 4* (Woting & Blaut, 2016).

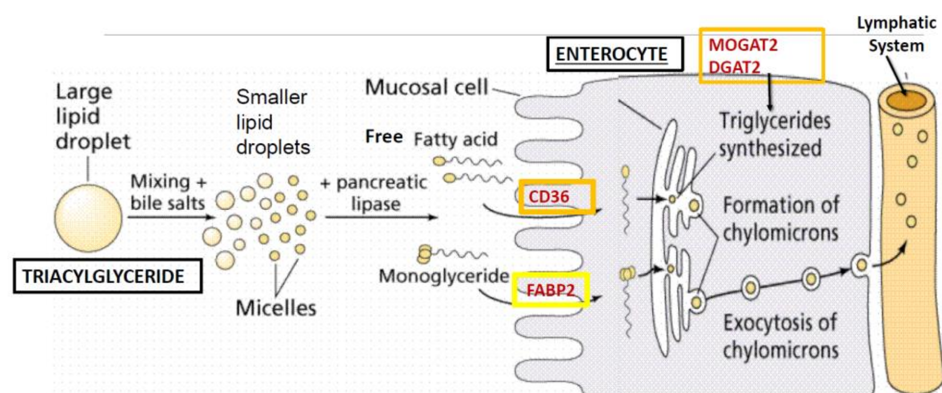

Figure 4 Lipid absorption in the small intestine. TAG is degraded by pancreatic lipase into FFA and MAG, which are further transported by CD36 or FABP2 through the brush-border membrane into the enterocyte. Once inside the enterocyte a two sequential acylation step is carried out by Mogat2 and Dgat2, in which TAG reforms. TAG is further packaged into chylomicrons and thereby travels into the lymphatic system for energy uptake. (Yen et al., 2015)

1.2.1.1 Genes involved in intestinal TAG synthesis

The absorption of FFA and MGAT into enterocytes requires FA transporters, such as CD36 the fatty acid translocase, fatty acid binding protein (FABP2) which catalyzes intracellular transport and FA esterification enzymes Monoacylglycerol O-Acyltransferase 2 (Mogat2) and Diacylglycerol O-Acyltransferases (Dgat1 and Dgat2) that are important for resynthesizing triglycerides for the formation into chylomicrons. (Gajda&Storch, 2015). FABP2 displays a high affinity binding for long chain fatty acids and other hydrophobic ligands, thus they are believed to be involved in uptake and trafficking of lipids in the intestine. Additionally, it was recently identified as a carrier of MAG.

The fatty acid translocase CD36 is involved in sensing long-chain fatty acids and might be a regulator of fat absorption at various stages, including the release of two gut hormones, secretin and cholecystokinin (cck) (Sundaresan et al., 2013) In a cell culture study it was found that the overexpression of CD36 promotes fatty acid (FA) uptake by increasing TAG synthesis, but not fatty acid transport across the plasma membrane. (Xu et al., 2013) The re-synthesis of TAG requires two sequential acylation steps carried out by Mogat2 and Dgat2, which takes place in the endoplasmic reticulum. The first acylation step is performed by Mogat2 which catalyses the synthesis of Diacylglycerol (DGAT), and uses FA-CoA thioesters as donors of acyl groups. The second acylation step is performed by Dgat2 that catalyses the TAG synthesis using DGAT and FA-CoA as substrates, see *Figure 5*. (Yen et al., 2015)

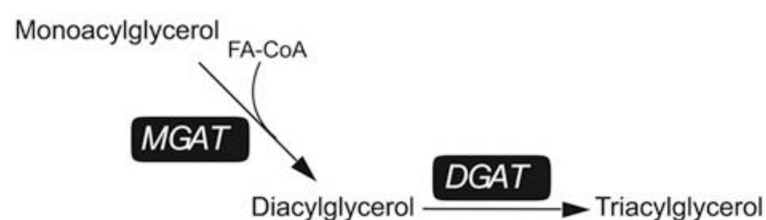

Figure 5 TAG synthesis pathway using MAG as initial acyl acceptor (Yen et al., 2015)

The nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor that regulates expression of genes involved in fat transport and storage, such as the FA translocase CD36. It acts primarily as a regulator of genes involved in fat metabolism.

When PPARγ is activated it promotes oxidation of long-chain fatty acyl CoA, and thereby protects from diet-induced obesity. (Rangwala et al., 2003) In contrast, mice lacking PPARγ in fat or liver developed hyperlipidemia (elevated levels of lipid in the blood) and hyperinsulinemia (elevated levels of insulin in the blood). Additionally, it was suggested to be a regulator of re-esterification enzymes. (Yen et al., 2015)

1.2.2 Gut microbiota promotes lipid absorption

That gut microbial composition effects lipid absorption and digestion was shown in a few studies. An impact of gut microbes on lipid absorption has been previously suggested but the mechanisms remain unknown. For example, studies by Rabot et al. demonstrated that GF mice on a HF diet excreted 40% more lipid in their faecal samples than their counterparts, although GF mice had reduced food intake compared to CONVD mice, see *Figure 6*. Thereby suggesting that GF mice are resistant to DIO. A trend in increased TAG and cholesterol values were found in fecal lipids of GF mice on HF diet, compared to CONVD mice on HF diet and CONVD mice on Chow diet (similar composition to LF diet). The increase in lipid absorption of CONVD mice led to an increased body weight compared to their GF counterparts, showing that GF mice are resistant to DIO (Rabot et al., 2010).

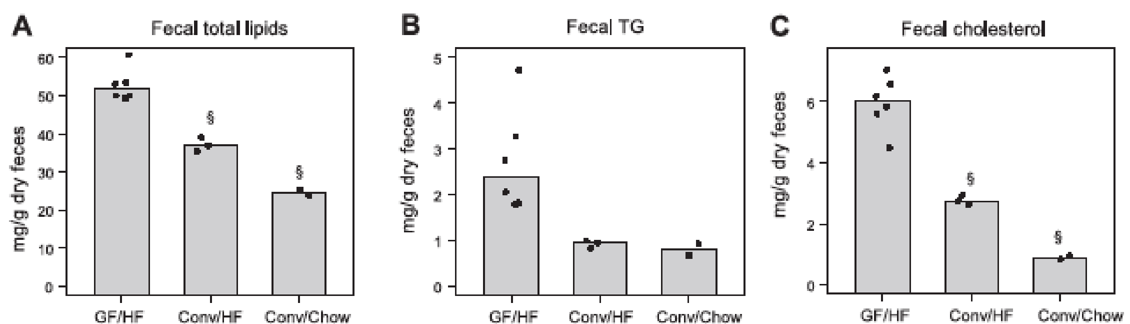

Figure 6 Comparison of feacal lipid excretion of GF on a HF diet and CONVD mice on HF diet or chow diet (high in fiber). (A) GF mice on a HF diet have increased lipid excretion by 40% in their faeces compared to their CONVD counterparts. Additionally.(B) Triacylglycerol (TG) and (C) Cholesterol was increased in faecal samples of GF mice on HF diet (Rabot et al., 2010).

Additionally, Rabot et al. demonstrated that mice lacking microbiota, effected diet-induced insulin sensitivity and cholesterol metabolism, see *Figure 7*. Measurements of insulin sensitivity and further oral glucose tolerance tests were performed. As expected CONVD mice on HF diet developed glucose intolerance and GF mice on HF diet had improved blood glucose levels and reduced insulin concentrations. However, the molecular mechanisms remain unclear (Rabot et al., 2010).

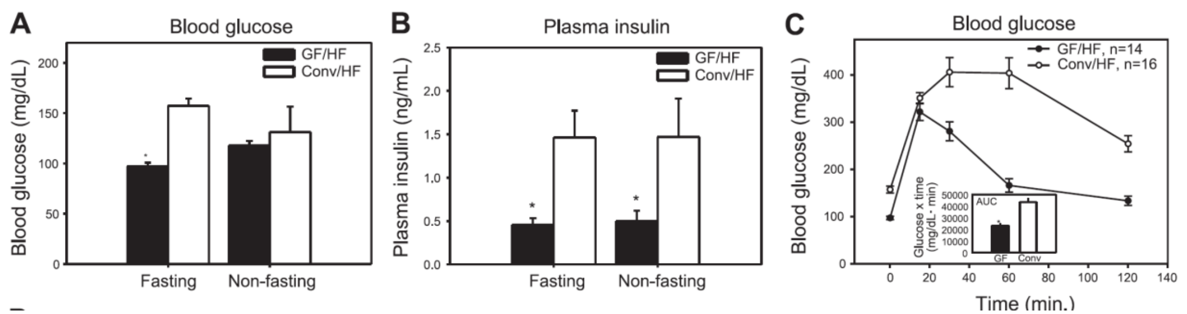

Figure 7 GF mice on HF diet are resistant to DIO and diet-induced Insulin sensitivity compared to their CONVD counterparts. (A) Blood glucose levels of GF and CONVD mice on HF diet were compared with GF and CONVD mice after 6 hours in a fasted state. In comparison to GF, the CONVD mice had elevated blood glucose level at 6 hours in a non-fed state, but not in the fed state. (B) Plasma insulin concentrations were significantly lower in GF on HF diet compared to CONVD mice. No impact of fasted vs. non-fasted state on plasma insulin concentration. Additionally (C) to examine insulin sensitivity of mice, oral glucose tolerance test were performed at the end of the study. The CONVD mice on HF diet developed glucose intolerance, wheres their GF counterparts had improved blood glucose tolerance (Rabot et al., 2010).

1.3 Clostridiaceae

Preliminary data from Eugene Chang's laboratory showed that a HF diet (rich in saturated milk-fat) reduces alpha diversity (measure of species richness) and increases the relative abundance of the family Clostridiaceae in the small intestine, as determined via 16S rRNA amplicon sequencing, see *Figure 8*. Members of the family Clostridiaceae, such as *Clostridium ramosum* has been suggested to have obesogenic effects.

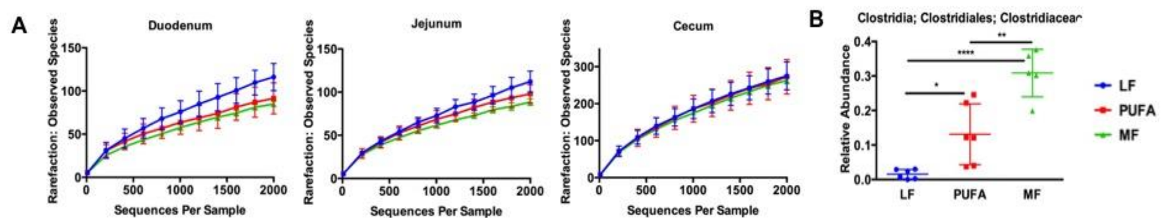

*Figure 8 High fat "Western" diets (MF – high in saturated milk fat diet; PUFA- polyunsaturated fat diet) reduces alpha diversity in the duodenum and jejunum compared to a low fat (LF) diet but not in the cecum (**A**). The MF diet significantly increases the relative abundance of the family Clostridiaceae in the jejunum compared to the LF diet (**B**). Data are shown as means +/- SEM (n=5-6). *P≤0.05; ** P≤0.01; ****P≤0.0001*

1.3.1 Obesogenic effect of *Clostridium ramosum*

Karlsson et al. investigated in women with T2D a correlation between increased abundance of *C. ramosum* in the gut and metabolic disorders (Karlsson et al, 2013). This finding was supported by Le Chatelier et al. by associating obesity with an increased abundance of *C. ramosum* and overall low bacterial gene content in human subjects (Le Chatelier et al, 2013). Therefore, *C.ramosum* may be a critical mediator in the development of obesity, besides other family members of Clostridiaceae.

A role for *C. ramosum* in obesity was further studied by Woting et al. who found an increased abundance of *C.ramosum* in colonized GF mice fed a HF diet. The research group developed three different testing groups of mice to investigate the obesogenic effect of *C.ramosum* all fed a HF diet. The first group of mice was associated with simplified human intestinal microbiota (SIHUMI) including *C.ramosum* the second group was associated with *C.ramosum* only and the third group was associated with SIHUMI without *C.ramosum.* In the two test groups that included

the bacterial species *C.ramosum* displayed increased body weight and body fat compared to SIHUMI without *C.ramosum, see Figure 9* (Woting et al., 2014).

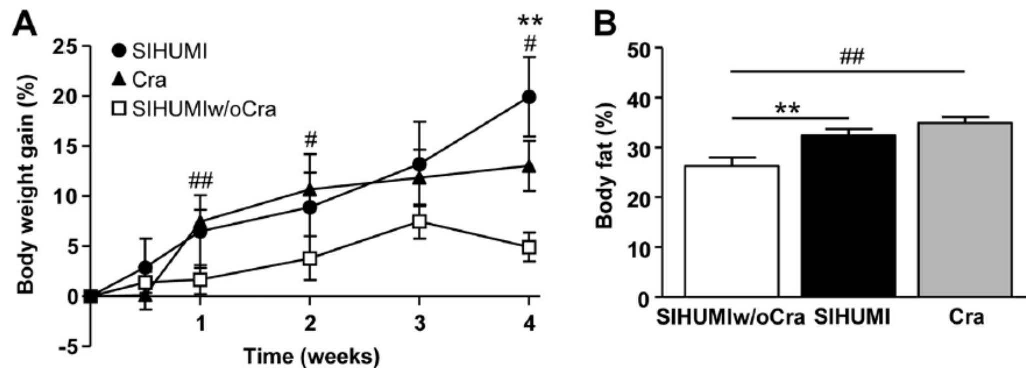

Figure 9 Presence of C. ramosum (Cra) promotes obesity in mice that harbour SI-HUMI and C.Ramosum only, compared to mice harbouring SIHUMI without C.ramosum. Mice were fed 4 weeks a HF diet. (A) The body weight gain percentage of SIHUMI mice is significantly higher than SIHUMI without C.ramosum (B) Mice harbouring C.ramosum have higher fat absorption, compared to SIHUMI mice without C.ramosum. n=8-9, for body fat n=3 (Woting et al., 2014)

Additionally, gene expression of CD36 was significantly increased compared to SI-HUMI without *C.ramosum,* see *Figure 10.* These findings suggest that *C.ramosum* increases gene expression involved in the small intestinal lipid absorption, also within microbial communities (Woting et al., 2014).

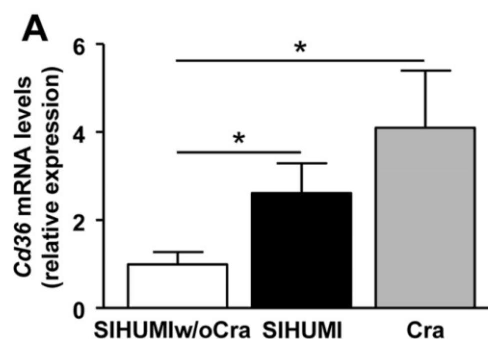

Figure 10 Impact of C.ramosum on liver metabolism was investigated by testing expression levels of genes involved in lipid transport, like CD36 (FA translocase), after 4 weeks on HF diet. The gene expression of CD36 in SIHUMI and C.ramosum only were significantly increased compared to SIHUMI without C.ramosum. (Woting et al., 2014)

1.3.2 Clostridium bifermentans

This spore-forming bacterium is anaerobic, gram-positive, and is commonly found in water, sewage and animal feces. It has pathogenic characteristics and has been associated with diseases such as osteomyelitis and necrotizing pneumonia. Besides fermenting carbohydrates including glucose, fructose and maltose, it is also able to produce metabolites such as lactate, acetate and ethanol. (Wong et al., 2014) A role of *C. bifermentans* in contributing to obesity or regulating small intestinal lipid absorption has not been described in previous studies.

Therefore, *C. bifermentans* was used in this study to determine its role in lipid absorption and transport.

1.4 Specific Aims

Preliminary results from Eugene Chang's laboratory show that HF diets increase the abundance of Clostridiaceae in the small intestine. To identify candidate strains of bacteria that might regulate lipid absorption, the 16S data set generated in Eugene Chang's laboratory from small intestine under LF and HF conditions was interrogated. Sequences belonging to the family Clostridiaceae were blasted by NCBI Microbial Nucleotide Blast® database (ref-NLM) and these sequences matched at a 96% similarity to *C. bifermentans*.

Therefore, the goal of this study was to determine if *C. bifermentans* promotes and/or induces lipid absorption and transport using *in vitro* (Aim 1) and *in vivo* (Aim 2) models.

1.4.1 Aim 1

The first goal was to determine *in vitro* if conditioned media from *C. bifermentans* increases fat uptake and lipid absorptive genes in small intestinal organoid cultures. We hypothesized that *C. bifermentans* increases genes that are involved in fat transport, esterification, as well as enteroendocrine genes like cholecystokinin and secretin. To test our hypothesis bacteria were grown under anaerobic conditions

and used for preparation of CM to treat duodenal and jejunal organoid cultures. CM from *C. ramosum* was selected as a positive control due to previous findings that this bacterium was found to be associated with obesity in humans (Le Chatelier et al. 2013) and promoted DIO in gnotobiotic mice and increased expression of CD36 in the small intestine (Woting et al. 2014), as previously mentioned. *Lactobacillus rhamosus gg* CM was selected as a negative control based on 16S sequencing results showing a reduction in Lactobacillales under HF diet conditions. In addition, time course experiments with 3H-oleic acid and 14C-monoacylglycerol were performed with monoassociated organoid cultures.

1.4.2 Aim 2

The second goal is to determine *in vivo* if *C. bifermentans* significantly contributes to host metabolism and obesity in antibiotic-treated C57BL/6 mice through its regulation of critical processes that are involved in lipid absorption and digestion. The digestive tract, physiology and anatomy, and the lipid metabolism pathways of mice are very similar to the human body, thereby serving as an ideal model to investigate cellular mechanisms involved in the development of obesity.

2 Materials and Methods

2.1 Generation and Culturing of Enteroids

2.1.1 Harvesting enteroids

The small intestine was removed from C57BL/6 mice (University of Chicago, Animal Facility) and was cut longitudinally with blunt-end scissors. The duodenal and jejunal tissue was rinsed several times in ice cold PBS (gibco, Life Technologies) and laid out with the luminal side facing up. With a glass slide, the luminal side was scraped gently to remove villi and mucous. Afterward the intestine was chopped with two razor blades into 1-2mm pieces and collected into a 50 mL conical tube containing 10 mL ice cold PBS. The pieces were agitated and rinsed, before aspirating the PBS, the tissue pieces had to settle down. Washing cycles were repeated 5 times or until the supernatant became clear. Then the intestinal pieces were resuspended in 25 mL ice cold 2.5 mM EDTA/PBS and rotated for 30 min at 4°C. Once the tissue has settled down, EDTA/PBS was removed and 10 mL Advanced DMEM/F12 (gibco, Life Technologies) added. Intestinal pieces were pipetted vigorously up and down. After the tissue settled down, the supernatant was collected into a fresh 50 mL conical tube. This step was repeated 3 times. In the next step the cells were isolated through a 70µm cell strainer. Then the cells were centrifuged at 300 rcf for 5 min at 4°C. The cell pellet was resuspended in 10 mL of Advanced DMEM/F12 and disrupted by pipetting up and down for at least 10 times. Afterward the cells were transferred into a 15 mL conical tube and centrifuged at 150rcf for 5 min at 4°C. This step was repeated 2-3 times until the supernatant became clear. In the last step 100 µL aliquots were mixed with 200 µL of Matrigel (BD Biosciences) and 50 µL of the mixture were plated onto a prewarmed 6-well culture plate (SIGMA, Corning Costar). It was important to work fast as the matrigel solidifies quickly at room temperature. The enteroid matrigel beads were allowed to settle for 1 hour in an incubator at 5% CO_2 at 37°C. After 1 hour, 2 mL of base media (DMEM/F12, Hepes, P/S, glutamine) including growth factors was added. Media was exchanged

every second day and the beads were split every 10-14 days, depending on the number and shape of the enteroids.

2.1.2 Passaging enteroids

The enteroids were split by pipetting up and down to separate the cells from matrigel, and by scraping gently on the surface of the well to remove the remaining enteroid-matrigel beads. Then the solution was pipetted into a 15 mL conical tube and centrifuged at 150g for 5 min at 4°C (Eppendorf). It is important that no cells are stuck in the matrigel. In that case the medium was removed so that 2 mL were left and additional 8 mL of Advanced DMEM/F12 were added. The solution was mixed by pipetting up and down and the previous centrifugation was repeated. Once all enteroids pelleted the medium was removed completely and fresh medium was added with the appropriate amount needed (such that the matrigel could be added at a 2:1 ration to the cell volume, to plate 50 µL beads). In the last step 100 µL of the cell aliquots were mixed with 200 µL of matrigel and 50 µL of the mixture were plated onto a prewarmed 6-well plate. The enteroid matrigel beads were placed for 1 hour in the incubator at 37°C, before the base media (DMEM/F12, Hepes, P/S, glutamine) including growth factors were added.

2.2 Treating generated Enteroids with conditioned media (CM)

Before the actual treatment of the enteroids, conditioned media of the four testing groups were prepared. Reinforced Clostridial Medium (RCM) Agar plates were prepared beforehand.

2.2.1 Preparation of RCM

In total 19 g of RCM (BD Biosciences) and 7.5 g of Difco Agar (BD Biosciences) were suspended in 0.5 L of RNAase-Free H_2O and mixed vigorously. Then it was autoclaved for 1 hour. Afterwards the autoclaved medium was poured into Petri Dishes (Falcon). Once the medium solidified the number of dishes needed were placed into the anaerobic chamber overnight.

2.2.2 Preparation of CM

On the next day the three bacteria to be tested including *Lactobacillus rhamnosus GG* (ATCC®53103™), *Clostridium ramosum* (ATCC®25582™) and *Clostridium bifermentans* (ATCC®638™), were streaked out and cultured for 1-3 days in the anaerobic chamber. Then a colony was picked from each plate and inoculated in 15 mL conical tubes with 10 mL RCM media, overnight. One tube without bacteria was prepared and served as control. Tubes were removed from the anaerobic chamber and spun down at 10000 rpm for 10 min to pellet bacteria. Afterwards the samples were sterile filtered and 500 µL were aliquoted into 600 µL tubes. The tubes were wrapped with parafilm and stored at -80°C.

2.2.3 Treating enteroids with conditioned media

Once enough enteroids were grown, they were split into a 24-well plate according to 2.1.2 and grown until 90% confluency. Afterwards duodenal and/or jejunal enteroids were treated with 10% conditioned media (CM) of the testing groups for 24h, as indicated in *Table 1*. Reinforced Clostridial Media (RCM) served as a vehicle control, Clostridium bifermentans (*C.bif*) was the testing group, Clostridium ramosum (*C.ram*) as a positive control and Lactobacillus rhamnosus gg as a negative control. *C.ram* was chosen as a positive control, due to previous studies by Woting et al., where it was shown that C.ram is associated with obesity and increased expression of cd36 (fatty acid translocase). In the case of Lgg, monoassociation studies showed no impact of Lgg on HF diet and lipid absorption marker gene expression in the small intestine, and is therefore serving as a negative control.

Table 1 Previously seeded enteroid beads were treated with 10 % of CM from C.bif, C.ram, Lgg and RCM as indicated in the table.

	A	B	C	D	E	F
1	***C.bif***	***C.bif***	***C.bif***	***C.bif***	***C.bif***	***C.bif***
2	***C.ram***	***C.ram***	***C.ram***	***C.ram***	***C.ram***	***C.ram***
3	***Lgg***	***Lgg***	***Lgg***	***Lgg***	***Lgg***	***Lgg***
4	**RCM**	**RCM**	**RCM**	**RCM**	**RCM**	**RCM**

After 24 hours the media was removed and 500 µL of cold PBS was added to silence the cells. Then the enteroids were harvested by pipetting up and down, and gently scraping the surface of the well. The solution from each well was transferred into a 1.7 mL microcentrifuge tube and centrifuged at 150g at 4°C for 5 min. Supernatant and matrigel was removed and 500 µL of Trizol (ambion, Life Technologies) were added to each tube. Tubes were frozen at -80°C until procedure with the RNA homogenization and isolation.

2.2.3.1 RNA homogenization and isolation

The samples containing 500 µL of Trizol were vortexed and homogonized with 1 mL syringe (BD Biosciences). An additional 500 µL of Trizol and 200 µL of Chloroform (Fisher) were added to the samples and shaked for 30 sec vigorously, followed by a 15 min centrifugation at 10000 rpm at 4°C. The top aqueous phase was transferred into a new set of tubes and mixed for 30 sec with 400 µL isopropanol (Fisher). The samples were incubated for 10 min. In case no pellet was visible, 0.5 µL of Glycogen (ambion, Life Technologies) were added to each solution and mixed by inverting. Samples were centrifuged at 10000 rpm for 10 min at 4°C. Afterwards the pellet was washed with 75% of ethanol. The RNA pellet was dried and depending on the pellet size 40-60 µL of Nuclease-Free Water (Ambion) were added and mixed gently until dissolvation of the pellet. The isolated RNA concentration was measured using the NanoDrop Lite (Thermo Scientific). RNA sample were stored at -80°C until further use.

2.2.3.2 cDNA synthesis

The RNA samples were diluted in Nuclease Free Water (Ambion) to a yield concentration of 1 µg/10 µL of total RNA. RNA dilution were transferred into 8-strip tubes (BioExpress) and a reverse transcription was performed by using the Transcriptor First Strand cDNA Synthesis Kit (Roche). 3µL of the Primer Master Mix were added to each tube and heated at 65°C for 10 min in the Thermalcycler (BioRad). Afterward the Master Mix was prepared and in total 7 µL were added to each tube, as indicated in *Table 2*.

Table 2 The Primer Master Mix (total of 3µL) and the Master Mix (total of 7µL) were prepared as indicated in the table.

Primer Master Mix	Amount Per Sample [µL]
Oligo dt primer	1
Random hexamer primer	2
Reaction Buffer	4
RNase Inhibitor	0.5
DNTPs	2
RT enzyme	0.5

The Real-Time (RT) reaction with the Master Mix of 7µL was heated in the Thermocycler for 10 min at 25 °C, 30 min at 55 °C, 10 min at 85 °C and finally at infinity hold at 4 °C. The cDNA synthesized samples were stored at -20°C until further use.

2.2.3.3 Quantitative Real-Time PCR

35 µL of the PCR Master Mix were added into a round bottom 96-well plate (Sigma Aldrich) and 2.5 µL per well of cDNA, giving a total volume of 37.5µL, as indicated in *Table 3*. The samples in the 96-well plate were mixed by pipetting and centrifuged at 1500 rpm for 1 min at 4°C. Each sample was transferred in triplicates of 10 µL into a flat bottom 384-well plate (Sigma Aldrich) and sealed with a Microseal® B adhesive sealer (BioRad). The 384-well plate was centrifuged at 1500 rpm for 1 min at 4°C and analyzed in the Light Cycler (Roche), with 45 Amplification cycles for 10 sec at 95°C, 20 sec at 56°C and finally for 30 sec at 60°C.

Table 3 A PCR Master Mix was prepared first, as indicated in the table.

PCR Master Mix	Amount per sample [µL]
Sybr Green (BioRad)	17.5
Nuclease Free Water (Ambion)	14
Forward Primer (IDT) (10µM working stock concentration)	1.75
Reverse Primer (IDT) (10µM working stock concentration)	1.75

Table 4 Forward and Reverse primer sequences used in qPCR analysis.

GeneTarget	**Forward Primer [5'-3']**	**Reverse Primer [5'-3']**
PPAR γ	GGA AGA CCA CTC GCA TTC CTT	GTA ATC AGC AAC CAT TGG GTC A
CD36	AGA TGA CGT GGC AAA GAA CAG	CCT TGG CTA GAT AAC GAA CTC TG
FABP2	TGC GAA CTG GAG ACC ATG AC	TCA GTC ACG GAC TTT ATG CCT
Mogat2	TGG GAG CGC AGG TTA CAG A	CAG GTG GCA TAC AGG ACA GA
Dgat1	TCC GTC CAG GGT GGT AGT G	TGA ACA AAG AAT CTT GCA GAC GA
Dgat2	GCG CTA CTT CCG AGA CTA CTT	GGG CCT TAT GCC AGG AAA CT
gapdh	GGC AAA TTC AAC GGC ACA GT	AGA TGG TGA TGG GCT TCG C
cck	TAC GAA TAC CCA TCG TAG TG	GTC GTA TGT GTG GTT GTT TC
secretin	TCA GAG TGG ACT GAA ACA AC	TAT TGA TGC CAA GGA CAA CC

2.3 Generation and culturing of Enteroid Monolayers

For generating duodenal or jejunal enteroid monolayers, 24 enteroid matrigel beads were needed to seed onto 24 monolayer transwells (Sigma Aldrich), 2.1.2 was followed. First, the transwells (24-well inserts, 0.33 cm^2 surface area, 0.4 µM pore polyester membrane) were coated with human collagen type IV solution (final concentration of 10µg/cm^2) and incubated at 37°C for a minimum of 2 hours. Afterward the coated transwells were washed 3 times with base media (DMEM/F12, Hepes, P/S, glutamine). After the last washing step 50 µL should remain. Enteroids were collected and washed as explained previously, and mechanically disrupted by pipetting at leat 30 times. Next, 50 µL of the disrupted enteroid mixtue was seeded onto the transwell coated with 100 µL collagen type IV solution and incubated for 1 hour at 37°C. In the last step 500 µL of base media (DMEM/F12, Hepes, P/S, glutamine) including growth factors was added onto and beneath the transwell. Media was changed carefully, to avoid disruption of the monolayer, every two days by removing and adding 450 µL.

2.3.1 Fatty Acid Uptake Assay

The enteroid monolayer was cultured until 90% confluency was reached for ideal conditions performing the FA uptake assay. Transwells coated with enteroids were treated for 24 or 48 hours with 10% CM of RCM, *C.bif*, *C.ram* and *Lgg* as indicated in *Table 1*. Time course experiments with 3H-oleic acid and 14C-monoacylglycerol were performed, after which a 10 min treatment time for duodenal enteroids and 15 min treatment time for jejunal enteroids was chosen for further experiments.

2.3.1.1 Preparation for the FA uptake assay

First of all theScintillation vials (Simport™ Scientific) were labeled appropriately. Followed by buffer preparation, see *Table 5*.

Table 5 The termination and Cell Lysis buffer were prepared accordingly

Termination Buffer	Ice cold 0.5% Bovine serum albumin (Sigma Aldrich) in 50 mL ice cold PBS was mixed and put on ice until needed.
Cell Lysis Buffer	0.1% Sodium dodecyl sulfate (SIGMA, Life Science) in 50 mL PBS was mixed and put into a warm water bath to ensure dis-solvation.

Stock solution of 3H-OA at 5µCi/µL and 14C-MAG at 0.25µCi/µL were prepared. In order to achieve a final concentration of 0.005 µCi/µL for 3H-OA and 14C-MAG a working stock at a concentration of 0.25uCi/ul was made up and diluted with PBS appropriately to the number of samples.

2.3.1.2 Performing FA uptake assay

10 µl of the working stock were added to wells accordingly to the time table. The media at the top and bottom of the transwell were removed prior the 3 washes, which were performed carefully with ice cold termination buffer not to disrupt the monolayer. Samples were taken from the media at the top and bottom of the transwell filter from 1 well per time point and each washing step. Termination buffer was removed completely after third wash and 0.5 mL Lysis buffer was added. Each well was scraped with the pipette tip and the solution was transferred into scintillation vials. Additionally, 250 µL of Lysis buffer was added to each well to ensure no remaining cells on the transwell filter.

2.3.1.3 After performing FA uptake assay

After the last harvest a swipe test was conducted. The radioactive waste was disposed accordingly to the Radiation Safety Guidelines.

Before the scintillation vials were transferred into the scintillation counter (Beckman LS6000), 4 mL of Scintillation cocktail (PerkinElmer) were added to each scintillation vial and mixed properly.

2.4 Animal Study

All experimental procedures with mice were approved by the University of Chicago Institutional Animal Care and Use Committee (IACUC). The C57Bl/6 mice were housed under standard 12:12 hour light/dark conditions at the University of Chicago. The age of the litter-matched specific pathogen free (SPF) male C57Bl/6 mice was between 8-10 weeks. They were either fed a purified low fat diet (Harlan Teklad 2018S) or a high saturated milk fat diet (Harlan Teklad TD.00102 customized diet) for the duration of the experiment.

2.4.1 Experimental Design

At the start of study mice (C57Bl/6J SPF), were 9-10 weeks old. The bedding was mixed across all mice cages 3 days prior to the introduction of the purified LF diet to ensure acclimation. The stool was collected at the baseline to ensure that all mice are starting with the same microbiota profile.

Table 6 Experimental design of the Animal Study. In total 20 mice were used and distributed into 4 different groups according to the table.

	Low Fat (LF)	**Milk Fat (MF)**
– *C.bif*	n=5	n=5
+ *C.bif*	n=5	n=5

After 4 days on LF diet, mice were depleted of gut bacteria by using a cocktail of antibiotics for 2 weeks, 250 mL per mouse. The cocktail contained 3 g of Metronidazole (1 mg/mL), 3 g of Ampicillin (1 mg/mL), Neomycin (1.5 mg/mL) and Vancomycin (1.5 mg/mL). Stool was collected 3 days after antibiotic treatment and weekly thereafter. With the completion of the antibiotic treatment, stool was collected and mice were transferred into a new cage and put on either LF or MF diet for 3 weeks. Two days after on the appropriate diet, mice were transferred into new cages (to ensure no residual antibiotics) before gavaged weekly with with *C. bifermentans* $1x10^9$ CFUs or saline control. The mice remained on diet with *C.bif* for up to 4 weeks or until notable changes were found in body weight. Stool was collected 3 days after

C. bifermentans gavage and weekly thereafter. Throughout the study food and body weight was measured bi-weekly, see *Figure 11.*

Figure 11 Experimental Design of the Animal Study, with all important steps conducted.

2.4.2 Establishing growth curve for *C.bifermentans*

On the first day the bacterial strain of *C. bifermentans* was streaked out in duplicates on RCM agar plates (prepared as described in 2.2.1) and grown for 3 days in the anaerobic chamber. A single colony was picked with an inoculation loop (Fisher 2-031-23) and grown in 10 mL RCM media overnight. On the following day hungate tubes (CLS-4209), syringes and needles (BD Biosciences) and petri dishes with RCM agar were put into the anaerobic chamber 1h prior start with the growth curve. 10 mL of RCM media and 200 µL of overnight *C. bifermentans* culture were added into two hungate tubes and additionally into a round-bottom tube for plating out later on. The two hungate tubes were removed from the chamber and the first reading was made, with the spectrophotometer at OD600 (Thermo Scientific, Spectronic 200). For each reading 500 µL were taken out with the following time points: 0, 0.5, 1, 1.5, 2, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5 until stationary phase was reached. The growth curve was plotted to identify the exponential growth, see *Figure 12*. When the culture reached the log phase, the hungate tubes were placed into the anaerobic chamber and 1 mL of bacterial solution from each hungate tube was removed for identifying bacteria. Additionally serial dilutions were prepared for plating out bacteria (100 µL/plate) to determine CFUs.

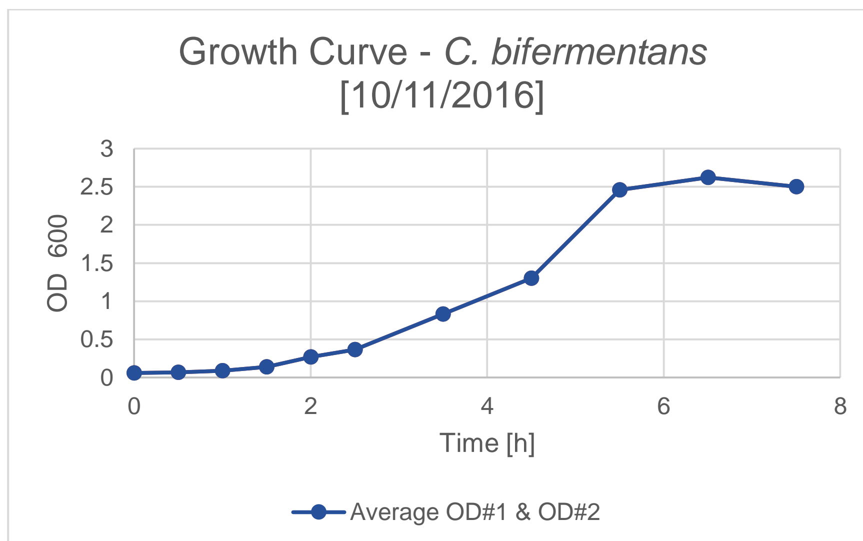

Figure 12 Establishment of the growth curve for Clostdridium bifermentans was consistent when repeated. Especially in the first 2h it grows fast, therefore reading were taken every 30 min. At approximately 6h the stationary phase was reached.

2.4.2.1 Verifying Bacterial Identity

1 mL aliquots were taken from each bacterial culture and centrifuged at max speed for 5 min at RT. The supernatant was removed. With an incoculation loop it was dipped into precipitate and mixed in a 8-strip PCR tube with 10 µL of Nuclease Free Water. The solution was microwaved for 2 min. The 16S Ex Taq PCR template was used, by using 25 µL of reaction including 2 µL of dirty lysate, as indicated in *Table 7.*

Table 7 For bacterial identity verification the 16S Ex Taq PCR template was used

Nuclease Free H20	17.375 µL
10x Buffer	2.5 µL
8F Forward Primer 5’ AGA GTT TGA TCC TGG CTC AG 3’	0.5 µL
1492 Reverse Primer 5’ GGT TAC CTT GTT ACG ACT T 3’	0.5 µL
dNTP	2.0 µL
ExTaq Enzyme (#RR001):	0.125 µL
Dirty Lysate	2.0 µL
Total amount	25 µL

The reaction with a total volume of 50 µL was heated in the Thermalcycler for 3 min at 95°C, followed by 34 amplification cycles for 30 sec at 95°C, 30 sec at 55°C, 1:30 min at 72°C, 5 min at 72°C and finally at 12°C infinite hold.

To identify if bacteria were present, 5 µL of the PCR reaction and a blank were transferred on 1% agarose gel in 0.5x TBE buffer. For successful amplification, 5 µL of the PCR reaction were transferred into a clean PCR tube and mixed with 2 µL of Exosap-IT. The reaction was incubated in the Thermocycler at 37°C for 15 min, at 80°C for 15 min and at 4°C indefinitely. Afterwards the samples were submitted for sequencing at the DNA Sequencing & Genotyping Facility from the University of Chicago. Once the sequence was established it was identified using the NCBI BLASTN for 16rRNA identification.

2.4.3 DNA extraction

The DNA was extracted from weekly collected fecal samples, and from cecal content for further 16S rRNA sequencing.

Approximately 250 µL of glass beads (0.1 mm dia., BioSpec) were added into a 2 mL screwcap Eppendorf tube (Thermo Scientific) with approximately 0.02g of fecal pellet. 1 mL of Lysis buffer and 20 µL of proteinase K were transferred to the tube. The Bead beater (Cell Disrupter 607, MIDSCI) was used to disrupt the pellet twice for 1min. Afterward the samples were incubated overnight in a 55°C water bath. The samples were removed from the water bath and centrifuged at max speed for 1 min at RT (room temperature). 600 µL of the supernatant were transferred into a new set of 1.7 mL microcentrifuge tubes and the equal volume of 600 µL from the top aqueous phase of Phenol Chloroform Isoamylalcohol (Ambion) was added. The tubes were shaked vigorously for 30 sec and centrifuged at max speed for 5 min at RT. 400 µL of the top phase was transferred into a new set of 1.7 mL microcentrifuge tubes and mixed with an equal volume of 400 µL chloroform. The tubes were mixed vigorously for 30 sec and centrifuged at max speed for 5 min a RT. A last time the top phase was removed into a new set of tubes and mixed by inverting with 100% Ethanol. The samples were placed for 30 min at -20°C to precipitate DNA and afterwards centrifuged at max speed for 5 min at 4°C. The pellet was washed with 70%

ethanol and dried at 55°C for 30 min with caps open. Depending on the size of the pellet it was resuspended in 50-70 µL Nuclease-Free water and placed with closed caps into the dryer at 55°C for 30 min to ensure resuspending of DNA. The DNA concentration was measured using NanoDrop Lite (Thermo Scientific). Samples were diluted to 20ng/µL for further 16S rRNA generation by an Illumina MiSeq DNA platform at the Argonne National Laboratory (Research laboratory operated by the University of Chicago)

2.4.4 Tissue collection

The mice were 15-16 weeks old, when they were carefully sacrificed with a sevoflurane overdose, followed by exsanguination and cervical dislocation for analyzing and measuring weight of gonadal fat, mesenteric fat, inguinal fat, retroperitoneal fat, liver, gallbladder and pancreas. Mucosal scrapings from the small intestine and cecal content were collected and immediately frozen in liquid nitrogen and stored at -80°C. Additionally blood was taken for analyzing lipids in the plasma. RNA was isolated from duodenal and jejunal tissue as described in 2.2.3.1. Using the cDNA Synthesis Kit (Roche) reverse transcription was performed (see 2.2.3.2) and further analysed with RT qPCR (see 2.2.3.3)

2.4.4.1 Triglyceride Assay

The Triglyceride level in plasma from mice was quantitatively determined using the WAKO L-Type Triglyceride Kit. First a layout was established for standards in duplicates. The standards were made up in 2-fold serial dilutions beginning with a stock standard concentration of 400 µL using water. Dilutions were loaded in duplicates. 5 µL of each plasma sample was loaded with 45 µL of nuclease-free water into the 96-well plate. 120 µL of Reagent 1 were added to all wells and mixed gently by pipetting. The 96-well plate was incubated for 5 min at 37°C. A blank reading was performed by using the Spectrophotometer at 600nm (Versamax, Microplate reader), before adding 40 µL of Reagent 2 to all wells. Then the actual reading was made using the spectrophotometer at 600nm.

2.4.4.2 SCFA extraction from cecal content

First, the cecal content was weighed (approximately 0.02g) and dispersed in 600 µL nuclease-free water with inoculating loops. Samples were vortexed and spun down at RT for 10 min at 13000g. 500 µL of the supernatent were transferred into a new set of microcentrifuge tubes and mixed with 100 µL of 50% sulfuric acid. Iso-butyric acid (IBA, SIGMA) was prepared (8.8 µL of IBA + 991.2 of nuclease-free water) and 5 µL of IBA were transferred into each tube, and vortexed. 500 µL of Ethyl Ether Anhydrous (Fisher) was transferred into each sample, vortexed and spun down at 13000g for 1 min at RT. The upper Ether layer was transferred into a glass vial (Fisher, clear 10-425 screw thread). The two previous steps were performed 2 more times. Then 250 µL of *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (Sigma-Aldrich) was added to each sample and incubated at RT overnight. The samples were run Gas Chromatography-Mass Spectrometry (Varian, CP 3800/Saturn 200) on the following day.

3 Results & Discussion

3.1 Conditioned media from *C. bifermentans* increases expression of genes involved in lipid absorption

Duodenal crypts were harvested from mice and cultured into duodenal organoids (enteroids)for three to four weeks, and treated with 10% CM for 24 hours with 4 treatment groups: *Clostridium bifermentans (C.bif)* as the experimental group, Reinforced Clostridial Media (RCM) as the vehicle control, *Lactobacillus rhamosus gg (Lgg)* as the negative control and *Clostridium ramosum (C.ram)* as a positive control. The CM experiments were repeated in jejunal enteroids, and similar trends appeared. For measuring genes involved in lipid absorption, the gene expression of mogat2, dgat1, dgat2, cck, secretin, cd36, fabp2 and pparγ was measured via RT-qPCR.

In the lipid absorptive pathway TAG is broken down by pancreatic lipase into FFA and MAG. The absorption is mediated by micelles. CD36 a fatty acid translocase transports the FFA into the enterocyte and the FABP2 acts as a carrier for MAG. Once inside the enterocyte, FFA and MAG are used as building blocks to re-synthesize TAG through two sequential acylation steps. The first acylation step is performed by Mogat2, followed by Dgat1 and Dgat2 enzyme reactions. TAGs are then packaged into chylomicrons and secreted into the lymphatic system to be utilized as an energy supply for the body. PPARγ acts as a signaling molecule that promotes fat storage in fat tissue, and is suggested to regulate re-esterification enzymes such as Mogat2, Dgat1 and Dgat2. Important regulators of lipid digestion are enteroendocrine hormones such as secretin (sct) and cholecystokinin (cck), as they promote the release of enzymes such as lipase to cleave FA from TAG for FA absorption through enterocytes.

It was found that at a 24 hour treatment with CM from *C. bifermentans,* the expression (transcription level) of re-esterification enzymes mogat2, dgat1 and dgat2 that are involved in lipid transport were selectively increased, as there was no effect on

other genes measured, such as CD36, sct, cck (important for FA transport and digestion). This may suggest that small molecules in *C. bifermentans* CM have a specific impact on absorption in the enterocytes but not on enteroendocrine signaling and digestion.

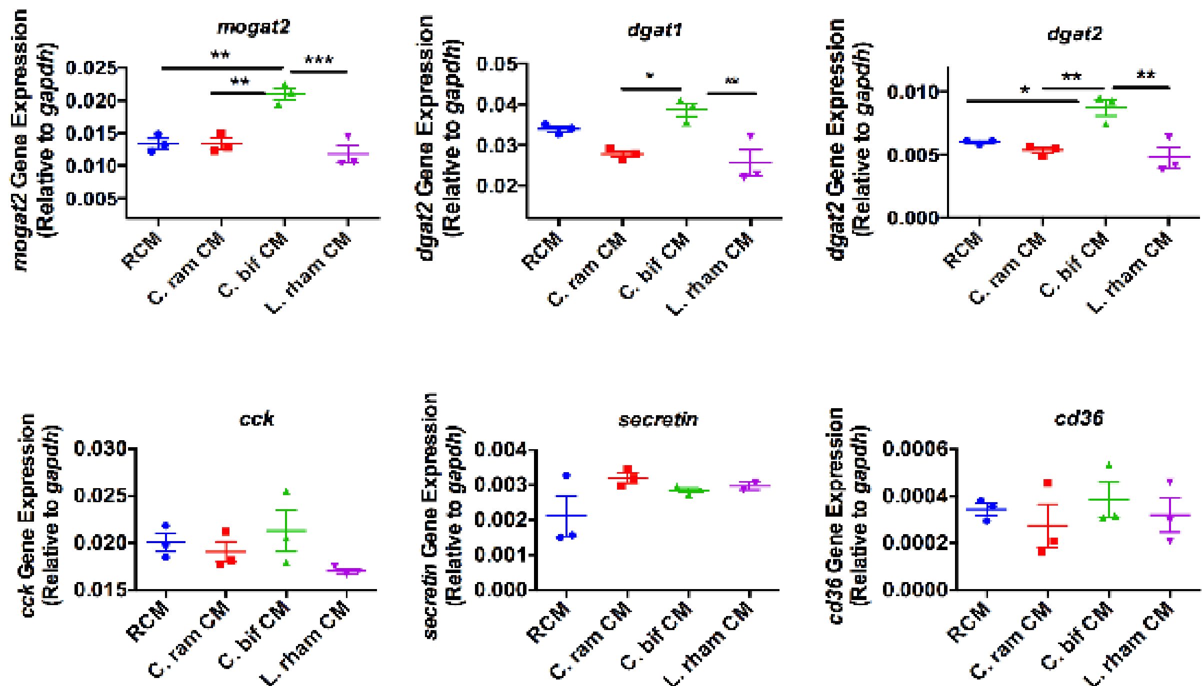

*Figure 13 Conditioned media (CM) from C.bifermentans (C.bif) increases gene expression of re-esterification enzymes mogat2, and dgat2 in duodenal enteroids compared to vehicle control RCM and CM from positive control C. ram and negative control Lgg at 24 h. Data are shown as mean +/- SEM (n=3). *P≤0.05; ** P≤0.01; *** P≤0.001*

3.2 Enteroid Monolayer Fatty Acid Uptake Assay

Duodenal enteroids were used to generate monolayers of intestinal enterocytes seeded into transwells by mechanical disruption and cultured for seven days. The monolayers were treated with 10% CM of RCM, *C.bif, C.ram* and *Lgg* for 24 hours prior treatment with 5μCi/μL of 3H-oleic acid. The 3H-OA uptake was measured in counts per minutes (CPMS) with the scintillation counter Beckman LS6000. Although not significant, a trend in increased 3H-oleic acid uptake with CM from *C. bifermentans* was observed. We concluded that while *C. bifermentans* has an effect on gene expression of re-esterification enzymes, it might not have a functional effect on the overall fat uptake and may require the involvement of other bacterial species.

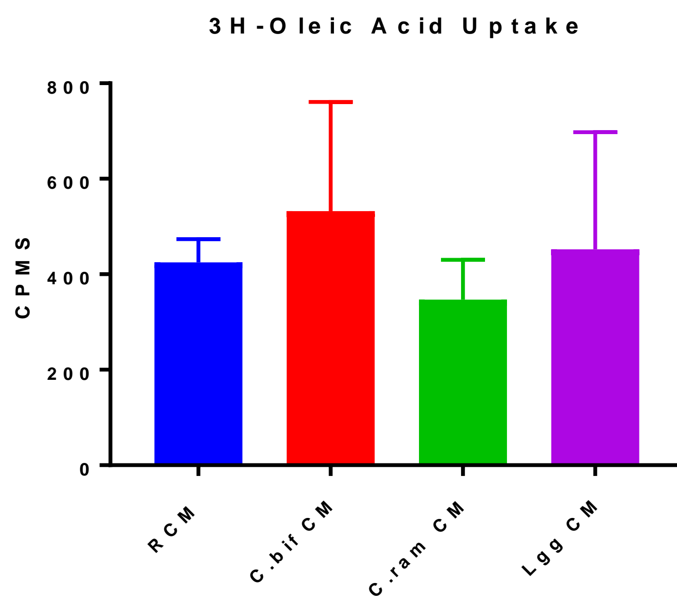

*Figure 14 Duodenal enteroids were grown for seven days and treated for 24h with 10% CM of RCM, C.bif, C.ram and Lgg , prior to treatment with 5µCi/µL of 3H-oleic acid. CM from C.bif has no significant effect on increasing 3H-oleic acid uptake, but a trend can be observed compared to C.ram CM, Lgg and RCM. Data are shown as mean +/- SEM (n=3). *P≤0.05; ** P≤0.01; *** P≤0.001*

3.3 Animal Study

3.3.1 Body weight & Food intake

To test the impact of *C. bifermentans in vivo*, C57Bl/6 mice were treated with an antibiotic cocktail for two weeks and then gavaged weekly with *C. bifermentans, w*hile the mice were fed either a LF or HF diet for three weeks. The HF diet increased overall murine body weight, but did not differ significantly in mice supplemented with *C. bifermentans,* see *Figure 15.*

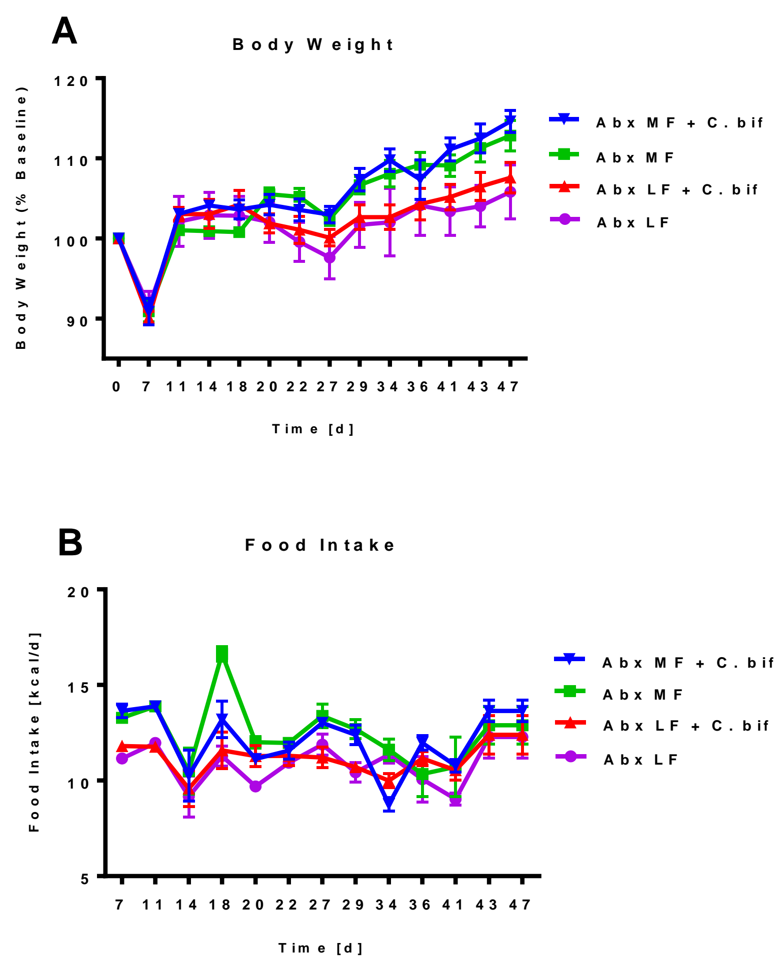

Figure 15 (A) No significant changes in body weight with C. bif supplemented subjects. Body weight was measured twice a week and mice were gavaged weekly with C.bif, after a 14-day course with antibiotics to remove microbes from the intestine for establishing a similar environment as in germ-free mice. (B) Food intake was measured bi-weekly.

A trend in increased body weight can be observed in mice supplemented with *C. bifermentans*, especially in the last week of the study. As the food intake is more or less constant within the four different groups, the change in body weight is likely not caused by different amounts of food intake.

3.3.2 *In vivo* supplementation with *C. bifermentans* increases expression of genes involved in lipid absorption

Mice supplemented with *C. bifermentans* displayed increased expression of lipid absorption genes in the small intestinal tissue of mice. The gene expression levels of isolated mRNA from mucosal scrapings of the duodenum were analysed.

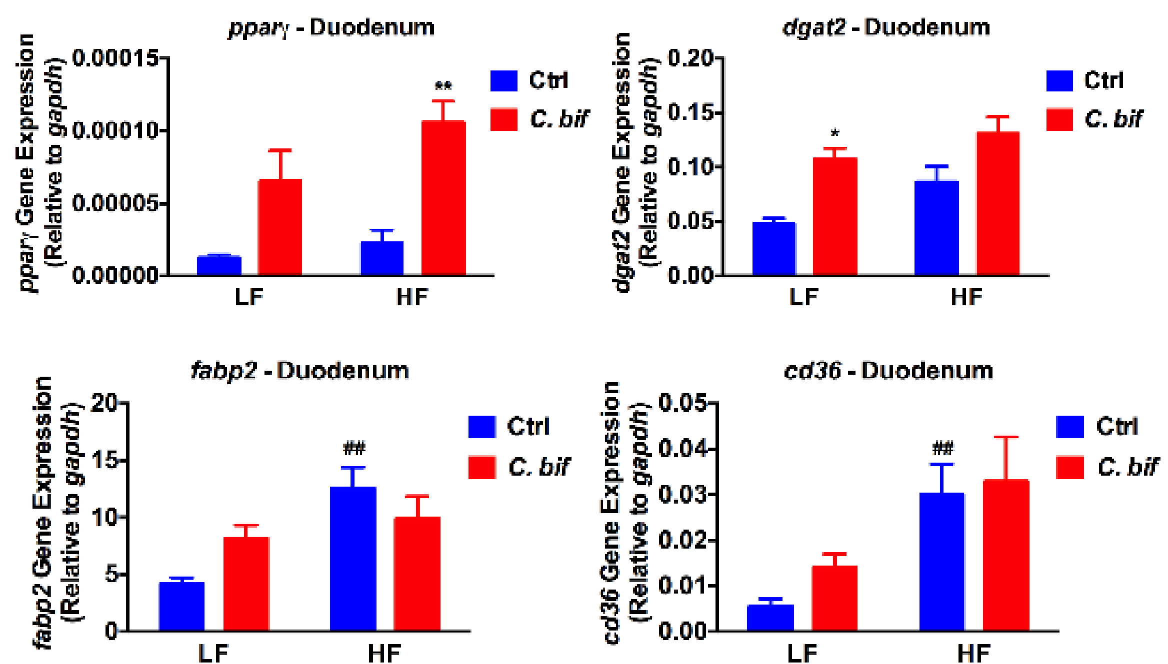

*Figure 16 Mice fed high fat (HF) or low fat (LF) diet following antibiotic treatment were supplemented without (Ctrl) or with C. bif weekly for 3 weeks. Gene expression of pparγ, dgat2, fabp2 and cd36 was measured in duodenal mucosal scrapings via qPCR. These graphs were analyzed with a two-way anova. Data are shown as mean +/- SEM (n=3-5). *P≤0.05; ** P≤0.01; *** P≤0.001*

Supplementation with *C. bifermentans* increased mRNA levels of PPARγ, a transcriptional regulator of genes involved in FA transport and storage, and Dgat2, which suggests a specific role of *C. bifermentans* in regulating lipid absorptive pathways, as PPARγ and Dgat2 seems to be influenced by the presence of *C. bifermentans* under LF and HF conditions.

There is a trend toward induction of FABP2 and CD36 expression by *C. bifermentans* under LF conditions, whereas under HF conditions FABP2 and CD36 are induced regardless of the presence of *C. bifermentans*. Similar trends in gene expression were observed in mucosal scrapings from the jejunum. Overal, these data suggest that *C. bifermentans* might be an important player in the promotion of fat absorption by upregulating re-esterification enzymes.

4 Conclusion and Future outlook

In conclusion, several studies demonstrated the role of gut microbiota in the development of obesity, but the functional aspect remain still a gap in knowledge. It is important to understand the mechanisms of microbe-mediated regulation of host metabolism. Through recent GF animal studies it was found that their resistance to DIO is caused by impaired lipid absorption and digestion in the upper GIT. (Bäckhed et al., 2007) SPF mice under HF diet have decreased alpha diversity of the gut microbiota and increased relative abundance of the bacterial family Clostridiaceae in the small intestine compared to SPF mice under LF diet. Operational taxonomic units that were elevated under HF conditions shared a 96% sequence similarity with *C.bifermentans*.

CM from *C.bifermentans* induced the expression of canonical re-esterification enzymes (Mogat2, Dgat1, Dgat2) that are important in lipid absorption in duodenal and jejunal enteroids. Additionally, duodenal mucosal scrapings of mice supplemented with *C. bifermentans* had increased gene expression levels of FABP2 and CD36 under LF diet conditions, but were elevated under HF diet regardless of supplementation. These findings suggest that members of Clostridiaceae might promote lipid absorption by triggering the upregulation of re-esterification enzymes.

Although the hypothesis was that *C. bifermentans* increases lipid absorption and contributed to the development of obesity, no significant changes in body weight or adiposity were observed. One potential explanation is that *C. bifermentans* might influence some parts of the lipid absorption pathway, like the expression of esterification enzymes, but other microbial community member are needed to impact other mechanisms driving lipid absorption. Another reason could be that the concentration of *C. bifermentans* supplementation was too low, on the other hand a higher concentration might have led to adverse outcomes.

Taken together, diet plays an important role in microbe-mediated interactions of small intestinal lipid absorption. By identifying the mechanisms and major players that are involved in the lipid absorptive pathway, we might find a way to treat obesity. In the fight against the worldwide epidemic, the controlled modulation of the gut

microbial structure and targeting metabolic pathways, might be the key in developing innovative therapeutic targets. In addition, it might be important to lower the abundance of Clostridiaceae and its members to reduce lipid absorption, and ultimately to prevent obesity and associated metabolic disorders.

List of References

Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI (2007) Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc Natl Acad Sci U S A.;104(3):979-84.;* doi:10.1073/pnas.0605374104.

Ballard O, Morrow AL (2013) Human Milk Composition: Nutrients and Bioactive Factors. *Pediatr Clin North Am. (1): 49–74.;* doi: 10.1016/j.pcl.2012.10.002.

Baothman OA, Zamzami MA, Taher I, Abubaker J; Abu-Farha M (2016) The role of Gut Microbiota in the development of obesity and Diabetes. *Lipids Health Dis.; 15: 108;* doi: 10.1186/s12944-016-0278-4.

Carmody RN, Gerber GK, Luevano JM, Gatti DM, Somes L,…Turnbaugh PJ (2015) Diet dominates host genotype in shaping the murine gut microbiota. *Cell Host Microbe.; 17(1): 72–84,* doi: 10.1016/j.chom.2014.11.010.

David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE,…Turnbaugh PJ (2014) Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014 Jan 23;505(7484):559-63; doi: 10.1038/nature12820.

De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB,…Lionetti P. (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci U S A. 107(33): 14691–14696.*; doi: 10.1073/pnas.1005963107.

Donaldson GP, Lee SM, Mazmanian SK (2016) Gut biogeography of the bacterial microbiota. *Nature Reviews Microbiology 14, 20-32;* doi:10.1038/nrmicro3552.

Gajda AM, Storch J (2015) Enterocyte fatty acid-binding proteins (FABPs): different functions of liver and intestinal FABPs in the intestine. Prostaglandins Leukot Essent Fatty Acids. 2015 Feb; 93:9-16. doi: 10.1016/j.plefa.2014.10.001.

Hartstra AV, Bouter KE, Bäckhed F, Nieuwdorp M (2015) Insights Into the Role of the Microbiome in Obesity and Type 2 Diabetes. *Diabetes Care 2015;38:159-165;* doi: 10.2337/dc14-0769.

Karlsson FH, Tremaroli V, Nookaew I, Bergström G, Behre CJ,…Bäckhed F. 2013. Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature 498:99–103.* http://dx.doi.org/10.1038/nature12198, 2013.

Kasubuchi M, Hasegawa S, Hiramatsu T, Ichimura A, Kimura I (2015) Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients. 2015 Apr 14;7(4):2839-49;* doi: 10.3390/nu7042839.

Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F,…Brandslund I (2013) Richness of human gut microbiome correlates with metabolic markers. *Nature 500:541–546;* doi:10.1038/nature12506.

Martin R, Nauta AJ, Ben Amor K, Knippels LM, Knol J, Garssen J (2010) Early life: gut microbiota and immune development in infancy.; Benefical microbes 1, 367-382; doi: 10.3920/BM2010.0027

Martinez KB, Pierre JF, Chang EB (2016) The Gut Microbiota : The Gateway to Improved Metabolism. *Gastroenterol Clin N,45 601–614.* http://dx.doi.org/10.1016/j.gtc.2016.07.001.

Rabot S, Membrez M, Bruneau A, Gérard P, Harach T,…Chou CJ (2010) Germ-free C57BL/6J mice are resistant to high-fat-diet-induced insulin resistance and have altered cholesterol metabolism. *FASEB J.;(12):4948-59;* doi: 10.1096/fj.10-164921.

Rangwala SM, Rhoades B, Shapiro JS, Rich AS, Kim JK,…Lazar MA (2003) Genetic modulation of PPARgamma phosphorylation regulates insulin sensitivity. Dev Cell. 2003 Oct;5(4):657-63.

Reuter J, Spacek DV, Snyder MP(2015) High-Throughput Sequencing Technologies. *Mol. Cell; (58), 586–597.* http://dx.doi.org/10.1016/j.molcel.2015.05.004.

Robinson S, Fall C (2012) Infant Nutrition and Later Health: A Review of Current Evidence. *Nutrients. (8): 859–874;* doi:10.3390/nu4080859.

Sekirov I, Russell SL, Antunes LC, Finlay BB. 2010. Gut Microbiota in Health and Disease. *Physiological Reviews 90 no. 3, 859-904, doi*: 10.1152/physrev.00045.2009 , 2010.

Sundaresan S, Shahid R, Riehl TE, Chandra R, Nassir F,…Abumrad NA (2013) CD36-dependent signaling mediates fatty acid-induced gut release of secretin and cholecystokinin; FASEB J. 2013 Mar; 27(3): 1191–1202. doi: 10.1096/fj.12-217703

Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI (2009) The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med.;1(6):6ra14;* doi: 10.1126/scitranslmed.3000322.

WHO. 2016. Obesity and overweight. http://www.who.int/mediacentre/factsheets/fs311/en/, 2016.

Wong YM, Juan JC, Gan HM, Austin CM. (2014) Draft Genome Sequence of Clostridium bifermentans Strain WYM, a Promising Biohydrogen Producer Isolated from Landfill Leachate Sludge. Genome Announc. 2014 Mar 6;2(2). pii: e00077-14. doi: 10.1128/genomeA.00077-14.

Woting A, Blaut M (2016) The Intestinal Microbiota in Metabolic Disease. *Nutrients; 8(4), 202.* 2016; doi:10.3390/nu8040202.

Woting A, Pfeiffer N, Loh G, Klaus S, Blaut M (2014) Clostridium ramosum Promotes High-Fat Diet-Induced Obesity in Gnotobiotic Mouse Models . *MBio.;(5):e01530-14;* doi: 10.1128/mBio.01530-14.

Xu S, Jay A, Brunaldi K, Huang N, Hamilton JA (2013) CD36 enhances fatty acid uptake by increasing the rate of intracellular esterification but not transport across the plasma membrane. Biochemistry. 2013 Oct 15;52(41):7254-61. doi: 10.1021/bi400914c

Yen CL, Nelson DW, Yen MI (2015) Intestinal triacylglycerol synthesis in fat absorption and systemic energy metabolism. J Lipid Res. 2015 Mar;56(3):489-501. doi: 10.1194/jlr.R052902.