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4. List of abbreviations

AAD	antibiotic - associated diarrhea
A.	*Akkermansia*
APS	ammonium Persulfat
BMI	body mass index
BSA.....	bovine Serum Albumin
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
Fiaf.....	fasting-induced adipocyte factor
F. prausnitzii	*Faecalibaterium prausntizii*
GC – content....	guanin/cytosin content
GIT.....	gastrointestinal tract
LB medium.....	lysogeny broth medium
LPL	lipoproteinlipase
LPS.....	lipopolysaccaride
NFW.....	nuclease free water
ob/ob.....	obese/obese
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rRNA.....	ribosomal ribonucleic acid
SCFA	short-chain fatty acids
TEMED	N,N,N',N' - Tetraethylmethylendiamin

TLR4 ..... toll like receptor 4

T-RFLP ..... terminal-restriction fragment length polymorphism

VLDL..... very low density lipoprotein

5. Summary

Over 10^^{14} microbial cells inhabit the gastrointestinal tract and about 1000 different species. The diversity and colonization differ between each person. Every individual has his own signature of the gut microbiota. This signature is depending on different factors. Age, nutrition, lifestyle, disease, inflammation as well as genetic factors play a role in the composition of the gut. The work focused on characteristics of microbiota in obesity and type 2 diabetes. The composition of the gut has influence on the energy extraction from the diet and as a consequence on body weight and the gut microbiota was analyzed in obesity and type 2 diabetes.

The aim of this study was to find out the differences of gut microbiota in obese and type 2 diabetics compared to lean control counterparts and the answer for estimating the possible role in controlling metabolic syndrome.

We focused on *Akkermansia muciniphila*, a mucin – degrading bacterium, *Prevotella*, known to design on Enterotypes, and *Faecalibacterium prausnitzii*, an indicator of inflammation or even diversity, in type 2 diabetes and obese patients. In group of twenty four insulin – dependent type 2 diabetes patients, fourteen obese participants with no established insulin resistance and nineteen healthy lean control, the gut microbiota was analyzed for abundance and diversity by quantitative real – time polymerase chain reaction, during gradient gel – electrophoresis and high throughput sequencing.

Especially the diversity differs between the groups. The highest diversity was found in lean patients, followed by obese participants and the lowest diversity in type 2 diabetes. There was also a difference between the bacterial groups. *Akkermansia*

are increased in type 2 diabetes. The observed enhanced abundance of *Akkermansia*, mucin degraders, in the diabetic group could indicate a better adaption on caloric restriction compared to the rest of the microbiota. *Akkermansia* are not depending on the nutrition of the host. *Prevotella* shows no significant difference between the Enterotype groups. *Faecalibacterium prausnitzii*, an indicator of inflammation, shows a decreased abundance in diabetes and obese persons.

Results were communicated in the following paper “Abundance and Diversity of microbiota in type 2 diabetes and obesity” published in Diabetes and Metabolism, March 2013, and in the paper “Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity”, submitted in PLOS ONE, April 2013.

6. Zusammenfassung

Mehr als 10^{14} mikrobielle Zellen besiedeln den Magen – Darm – Trakt. Die Vielfalt und Kolonisation der über 1000 verschiedenen Spezies sind von Person zu Person verschieden. Jeder Mensch hat seine eigene Unterschrift der Mikrobiota. Diese Signatur ist von verschiedenen Faktoren abhängig. Alter, Ernährung, Lebensstil, Krankheiten, Entzündungen sowie genetische Faktoren spielen bei der Zusammensetzung der Mikrobiota eine Rolle. Die Arbeit konzentrierte sich auf die Änderungen der Mikrobiota bei Personen mit Übergewicht und Type 2 Diabetes. Die Zusammensetzung des Darms hat Einfluss auf die Energiegewinnung der Ernährung sowie auf das Körpergewicht.

Das Ziel dieser Studie war es die Unterschiede sowie Änderung der Zusammensetzung der Mikrobiota bei Übergewichtigen und Type 2 Diabetiker/innen verglichen zu normalgewichtigen Personen zu untersuchen und eine Antwort auf die Abschätzung der möglichen Rolle auf die Kontrolle des metabolischen Syndroms zu finden.

Die Schwerpunkte dieser Arbeit waren die Bestimmung von *Akkermansia muciniphila*, ein mucin – abbauendes Bacterium, *Prevotella*, ein bekannter Enterotype, sowie *Faecalibacterium prausnitzii*, ein Indikator für Entzündungen oder auch Diversität, in Diabetes Typ 2 und übergewichtigen Personen. An der Studie nahmen 24 Insulin – abhängige Diabetiker/innen, 14 übergewichtige Personen, die keine Insulinresistenz aufwiesen, und 19 normalgewichtige Patienten, als Kontrollgruppe, teil. Die Diversität und Häufigkeit der Darmmikrobiota wurde mittels real – time Polymerase Kettenreaktion (PCR), sowie Gradienten Gel – Elektrophorese und high throughput Sequencing ermittelt.

Besonders die Diversität ändert sich zwischen den drei Gruppen. Die höchste Vielfalt an Bakterien zeigten normalgewichtige Personen, gefolgt von den übergewichtigen Teilnehmern/innen. Die niedrigste Abwechslung an der Mannigfaltigkeit der Besiedlung von Bakterien im Darm wiesen Type 2 Diabetiker/innen auf. Das Vorkommen von *Akkermansia* ist bei Typ 2 Diabetikern erhöht. Die beobachtete erhöhte Menge von *Akkermansia* in dieser Gruppe könnte auf eine bessere Anpassung an kalorische Restriktion im Vergleich zum Rest der Mikrobiota hindeuten. *Prevotella* zeigte keinen signifikanten Unterschied zwischen den Enterotype Gruppen. *Faecalibacterium prausnitzii*, ein Indikator für Entzündungen, zeigte eine verminderte Häufigkeit in Personen mit Diabetes und Adipositas.

Die Resultate der Studie sind im Paper "Abundance and Diversity of microbiota in type 2 diabetes and obesity", veröffentlicht in Diabetes and Metabolism, März 2013, und im Paper „Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity“, eingereicht in PLOS ONE, April 2013, zusammengefasst.

7. Introduction

7.1. Colonization of the Gut

Human gut microbiota plays an important role in digestion and metabolism. It is essential for a normal gut physiology as well as for health and influence the brain – gut communication (Aziz, Doré et al. 2013).

Microbiota includes all three domains of life – Bacteria, Archea and Eukarya (Bäckhed, Ley et al. 2005). In humans, approximately $10^{\wedge 14}$ microorganisms colonize the surface of skin, the gastrointestinal tract, genital and also the respiratory tract (Harris, Kassis et al. 2012). This includes about 1.000 different species. The *Bacteroidetes* and the *Firmicutes* are the two major phyla in the gut, followed by the *Actinobacteria* and the *Proteobacteria* (Cani and Delzenne 2009).

The largest number of bacteria resides the intestine and affects physiological processes in the body. The extraction of nutrients and the energy production from the diet play a decisive role for the digestion and also influence the gastrointestinal tract. Nutrition is not the only factor which is important for the development of a healthy microbiota. Other factors can influence the intestinal flora. Antibiotics, environment (stress), breast feeding, birth (vaginal or Caesarean section), body weight, age and a lot of other parameters can influence the microbial colonization in the gut and also the diversity of the bacteria. The stability and also the variety alter with increasing age (Lozupone, Stombaugh et al. 2012).

Each person has his own signature of microorganisms in the gut – from more frequent settlement till special bacteria strains. This colonization can change because of life style (nutrition) or disease (Aziz, Doré et al. 2013). The dominate bacteria

phyla are: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* as well as *Archea* (*Euryarchaeota*). *Firmicutes* include *Lactobacillus*, *Clostridium* and *Ruminococcus*, but also the butyrate producers *Eubacterium*, *Faecalibacterium* and *Roseburia* belong to this phylum. The phylum *Bacteroides* play an important role in the defense of pathogen bacteria. *Bacteroidetes*, *Prevotella* and *Xylanibacter* degrade a multiple number of complex glycan (polysaccharide). *Actinobacteria* include *Bifidobacteria*, which are proposed to have a probiotic effect. *Proteobacteria*, *Escherichia* and *Desulfovibrio*, are sulfate reducing bacteria. *Akkermansia* is part of the *Verrumicrobia*. This kind of species helps to degrade mucus (Tremaroli and Bäckhed 2012).

After birth, in the first three days, there is a primary colonization of *Bifidobacteria* (Ottman, Smidt et al. 2012). Afterwards, an ongoing composition and growth starts until a stable plateau is reached (Lozupone, Stombaugh et al. 2012).

7.2. Obesity and Diabetes mellitus type 2

Overweight and diabetes mellitus type 2 are very common in our population. In Austria currently about 390.000 people live with diabetes. Due to the lack of blood glucose screening the actual number is much higher. In many patients, the diabetes is only discovered after the occurrence of a diabetes-related complication. Overall, there are about 390.000 diabetics in Austria, but only 10% of all diabetes cases attributed to type 1. Type 2 diabetes occurs much more frequently in the context of obesity, lack of exercise or genetic predisposition. Patients with suffer from type 2 diabetes may also have increased blood fat, blood pressure and uric acid - metabolic syndrome (statistics Austria, 2007).

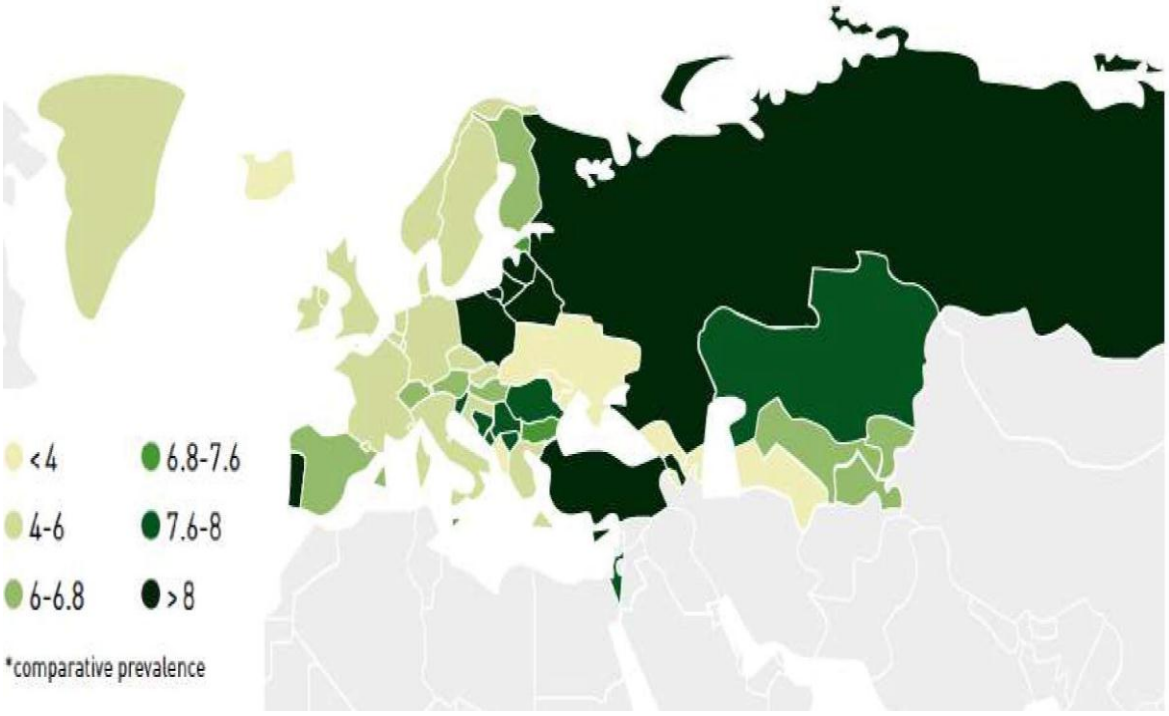

Figure 1 Prevalence*(%) estimates of diabetes (20 – 79 years), 2011, Europe Region (Ärztekammer Wien)

Obesity is a complex process and genetically and environmental factors are involved (Musso, Gambino et al. 2010). In many cases, an increased consummation of energy rich food and a sedentary lifestyle are associated with overweight. Obesity comorbidities are coronary heart disease, hypertension and stroke but also diabetes mellitus is a consequence of enhanced body weight (Harris, Kassis et al. 2012).

BMI (body mass index)	**Classification**
< 18.5	Underweight
18.5 – 24.9	Normal weight
25.0 – 29.9	Overweight
30.0 – 34.9	Class I obesity
35.0 – 39.9	Class II obesity
≥40.0	Class III obesity

Table 1: Classification of obesity (Pi-Sunyer 2000)

Diabetes type 2 is a result of insulin resistance and is marked by high blood glucose. A sedentary lifestyle and dietary factors influence the risk of developing diabetes. It is a metabolic disease and the utilization of sugar is inhibited. In most cases Obesity is the common reason for developing diabetes mellitus type 2. The therapy of diabetes is weight reduction and movement. If this general steps are not successful, different medicaments (tablets) and insulin are the second choice.

condition	*2 hour glucose* mmol/l (mg/dl)	*fasting glucose* mmol/l (mg/dl)	*HbA1c* %
normal	< 7.8 (< 140)	< 6,1 (<110)	< 6.0
impaired fasting glycaemia	< 7.8 (< 140)	≥ 6.1 (≥ 110) and 7.0 (< 126)	6.0 – 6.4
impaired glucose tolerance	≥ 7.8 (≥ 140)	< 7.0 (< 126)	6.0 – 6.4
Diabetes mellitus	≥ 11.1 (≥ 200)	≥ 7.0 (≥ 126)	≥ 6.5

Table 2: Classification of diabetes mellitus (Craig, Hattersley et al. 2009)

7.2.1. Obesity, Type 2 Diabetes and Microbiota

The gut microbiota is an important tool in obesity and type 2 diabetes. The intestinal gut affects the host metabolism by increasing the energy extraction, immune system modulation and modification of the lipid metabolism. Gut microbiota is also an important parameter in energy balance and obesity, associated with diabetes. The body weight is modified according to gut composition, nutritional ingredients (Harris, Kassis et al. 2012) and the energy harvest from the diet. Another crucial factor is the host energy homeostasis in the regulation of the intestine microbiota (Musso, Gambino et al. 2010).

Obesity is associated with an increased number of *Firmicutes* and a decreased of *Bacteroidetes* (Qin, Li et al. 2012). The ratio *Firmicutes* to *Bacteroidetes* is significant and positive correlated to reduce glucose tolerance, but negatively to a higher body mass index (Larsen, Vogensen et al. 2010).

Also the function alters during obesity and diabetes. The development of obesity in leptin - deficiency ob/ob mice moves the abundance of two domain bacteria phyla in another trend. Compared to the lean litter mattes, obese mice show a reduction in the wealth of Bacteroidetes and a proportional increase in Firmicutes. Both, obese and lean mice, are fed with nutrition rich in polysaccharide (Ley, Bäckhed et al. 2005).

The gut microbiota is a factor, which regulates the fat storage. In other animal experiments, germ – free and conventional raised mice are fed with high – fat, carbohydrate rich Western diet. After eight weeks, germ – free mice showed a lower body weight and fat mass development than the conventional raised mice. Germ –

free mice have a protective impact against the Western diet induced glucose intolerance and insulin resistance. An interesting point in this animal study was the energy content in the feces.

Both types of mice had similar energy – content in their feces. This indicates that an efficient energy - yield from the nutrition is not the only factor, which gains the fat mass at conventional mice (Bäckhed, Manchester et al. 2007).

7.2.2. Metabolism and Microbiota

Animal studies help to understand the connection between the metabolism (host metabolism) and the gut microbiota (Cani and Delzenne 2009).

Conventionalization doubled the density of small intestinal villi capillaries and improved the absorption of monosaccharide from the gut in the portal blood, stimulated the element binding proteins and supported the fat accumulation in the liver and fat tissue (Musso, Gambino et al. 2010).

Another factor which plays an important role between metabolism and microbiota is the fasting – induced adipose factor (FIAF), a member of the Angiopoietin – like family of proteins. This analyzes of germ – free mice, normal and FIAF knockout – mice have shown, that a circulating FIAF is an inhibitor of lipoprotein – lipase. Another term is Angiopoietin – like protein 4 (ANGPTL4). Under hypoxic conditions FIAF is induced in endothelium cells and is the aim of peroxisomes proliferation activators receptors. The coded protein, a serum – hormone, is directly involved in the regulation of the glucose concentration, lipid metabolism and insulin resistance.

This factor also appeared as an apoptosis survival factor for vascular endothelium cells (Bäckhed, Manchester et al. 2007).

FIAF is an extreme powerful regulator of the fat metabolism and obesity. An over-expression of FIAF caused a 50% reduction of the fat storage and weight, partly by stimulation of the fatty acid oxidation. Plasma levels of triglycerides, free fatty acids, glycerin, total cholesterol as well as high density lipoprotein cholesterol (HDL) are increased during a FIAF overexpression. The effects of a FIAF overexpression are enhanced by a diet which is high in fat. This factor also has consequences to plasma and lever triglyceride levels, free fatty acids and plasma glycerin. FIAF transgene mice who were feed with a fatty diet showed also a disordered glucose tolerance (Mandard, Zandbergen et al. 2006). Reduced expression of the protein is associated with diabetes type 2 (Bäckhed, Manchester et al. 2007).

Germ – free mice demonstrate an improved activity of the liver; also in muscle cells by oxidation of fatty acids pathways. These are mediate by the enzyme AMP – activate protein kinase as a result of enhanced activity. The enzyme plays a key role in the mitochondrial fatty acid oxidation, including Acetyl CoA Carboxylase and carnitine palmitoyltransferase i (Musso, Gambino et al. 2010).

Another triggering factor is the bacterial Lipopolysaccharide (LPS), produced by gram – negative bacteria. The transport pathways of LPS in the blood are the chylomicrons (Lipoprotein). LPS is an inflammation factor inducing the insulin resistance, metabolic endotoxemia, inflammation, obesity and diabetes. A diet which is rich in fat, results increased LPS – levels. The CD14 co - receptor binds LPS and together with TLR – 4, they have a pro – inflammatory immune response. A deficiency of the co – receptor is protective. Also a diet high in fat increased the LPS –

levels. Reducing the plasma – LPS – concentration could be a strategy to fight against metabolic disorders (Cani, Amar et al. 2007).

Short chain fatty acids are produced by fermentation of dietary fiber. SCFA not only affect as energy substrate for the host but they are also signaling molecules. They are ligands for two G-protein coupled receptors, GPR 41 and GPR 43 (Cani and Delzenne 2009). An animal study with gnotobiotic and conventional mice indicates, that a GPR41 deficiency is combined with a decrease expression of PYY. PYY is an enteroendocrine associated cell – derived hormone that normally inhibit the gut mortality, increases the gut function and reduces the energy harvest from the diet. These results suggest, that GPR 41 is a regulator for the host energy balance which effects are depending on the gut microbiota (Samuel, Shaito et al. 2008).

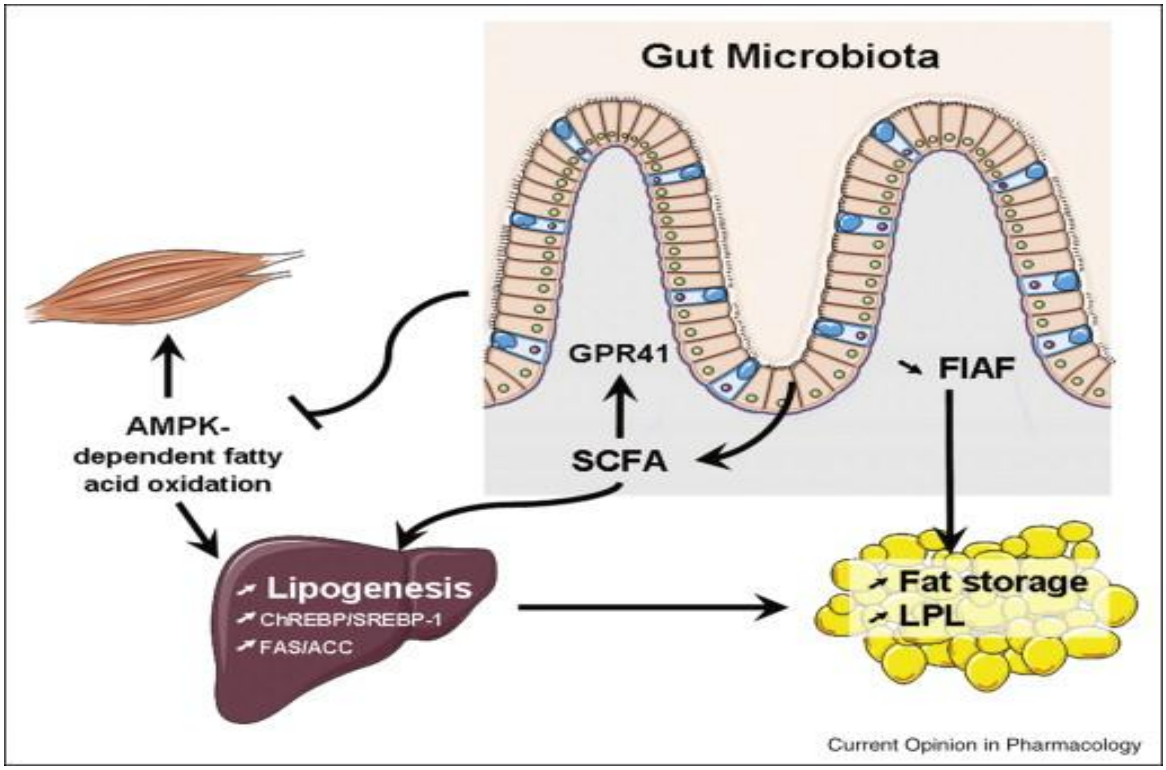

Figure 2: Gut microbiota harvest energy from the diet increases energy storage (Cani and Delzenne 2009)

The above points indicate that the gut microbiota can modulate the energy balance as well as the energy harvest from the diet, the energy storage by triglyceride and by oxidation of the fatty acids. This is maybe a connection between diet induced obesity, insulin resistance and diabetes.

In my master thesis, I focused on *Prevotella*, *Akkermansia* and *Faecalibacterium* and their relationship to obesity and diabetes type 2. The literature indicates that *Prevotella* is increasing in participants with obesity and diabetes compared to their lean counterparts (Larsen, Vogensen et al. 2010) and correlates negatively with energy intake (Tremaroli and Bäckhed 2012).

Akkermansia shows no significant differences between non – diabetic and diabetic person (Larsen, Vogensen et al. 2010).

F. prausnitzii is not really present in obese and diabetic persons, but increase after a surgery (bypass – operation). Levels of *F. prausnitzii* are negatively correlated with inflammatory markers (Tremaroli and Bäckhed 2012).

7.3. Main Enterotypes

Arumugam et al. sequenced the DNA of thirty nine - spot tests and compared them with a reference Genome. The participants of this study came from six different nations. They indicated three Enterotypes as common in the human gut microbiota (Arumugam, Raes et al. 2011), independent of the origin, nutrition, age or gender of the test person.

Also a ten day dietary intervention was not enough to change the settlement of the Enterotypes in the human gut microbiota, which indicate that a longer modification

is necessary to influence or alter the composition of the gut microbiota (Wu, Chen et al. 2011).

According to the type of bacteria, they built different clusters with specific characters, in which differ taxonomic bacteria species dominate. These include:

- Enterotype 1: *Bacteroides*
- Enterotype 2: *Prevotella*
- Enterotype 3: *Ruminococcus*.

These three Enterotypes use different ways to generate energy from the substrate and provide these individually to the host. Due to the bacterial composition and the variation of the functionality, each Enterotype adapt optimally the energy supply to the host.

- Enterotype 1 is rich in *Bacteroides* and gains their energy principally by fermentation of carbohydrates and proteins.
- *Prevotella* and *Desulfovibrio* dominate in the Enterotype 2, which are involved in the metabolism of proteins. *Prevotella* also belong to the mucin - degrading bacteria.
- *Ruminococcus* and *Akkermansia* control the Enterotype 3. This type of bacteria is engaged in splitting sugar and mucin (Arumugam, Raes et al. 2011).

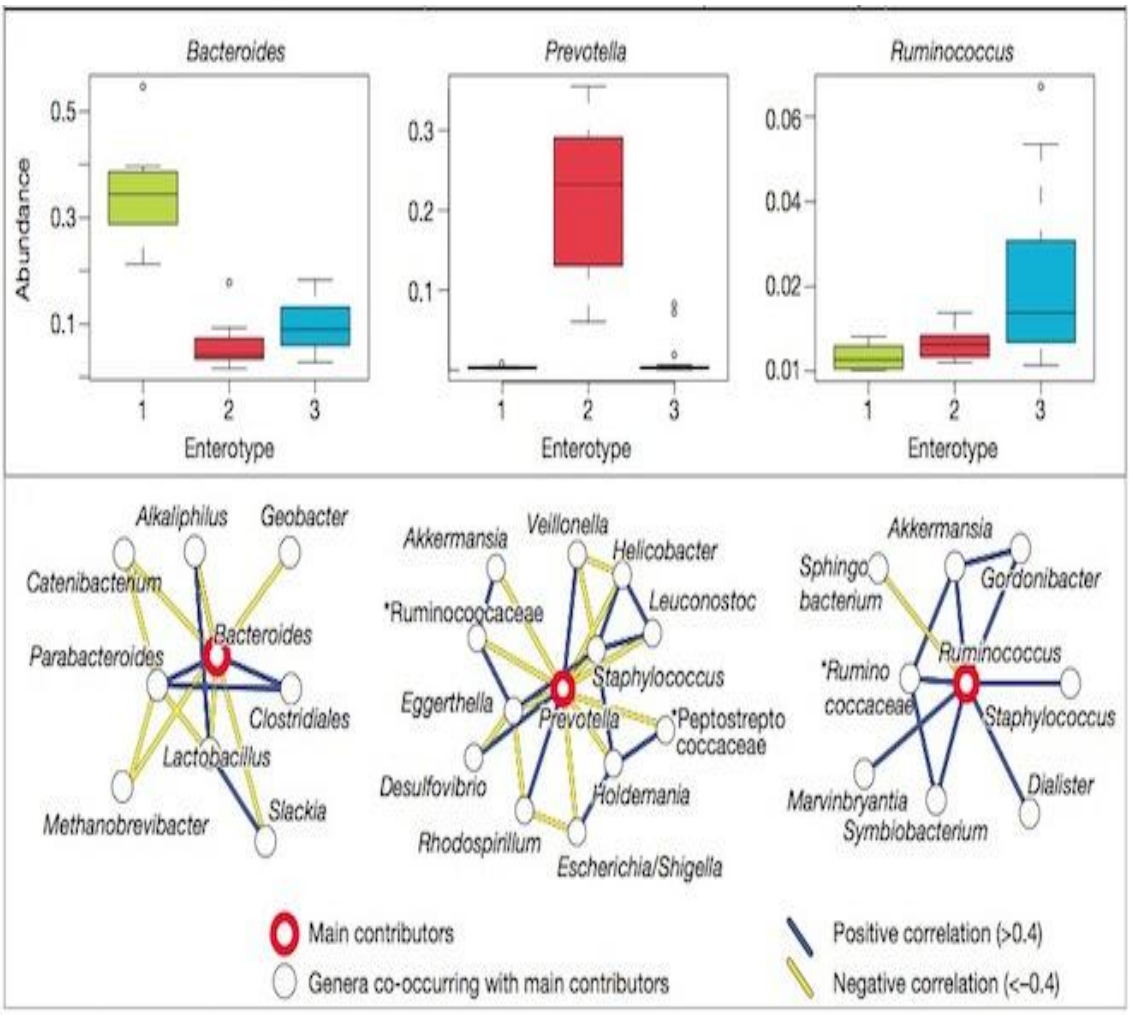

Figure 3: Phylogenetic difference in Enterotypes (Arumugam, Raes et al. 2011)

7.4. *Prevotella*, *Akkermansia* and *Faecalibacterium*

The composition of the gut is characterized by different bacterial groups and is a complex system. In my master study important classes are included:

- *Prevotella*,
- *Akkermansia* and
- *Faecalibacterium*.

7.4.1. *Prevotella*

Kingdom	Bacteria
Phylum	Bacteroidetes
Class	Bacteroidetes
Order	Bacteroidales
Family	Prevotellaceae
Genus	Prevotella
Type species in our study (primer detection)	p.falsenii p.copri, p.nigrescens., p.intermedia., p.pallens., p.maculosa., p.oris (Larsen et al., 2010)

Table 3: Classification of the genus *Prevotella*

Prevotella belongs to the gram – negative bacteria and settles the intestinal tract. *Prevotella* is also common in the vaginal and oral flora. Different types colonize the body and influence through variable pathways.

Infections of *Prevotella* can affect the respiratory tract and there is also a connection between periodontitis and periodontal abscess. (Tanaka, Yoshida et al. 2008).

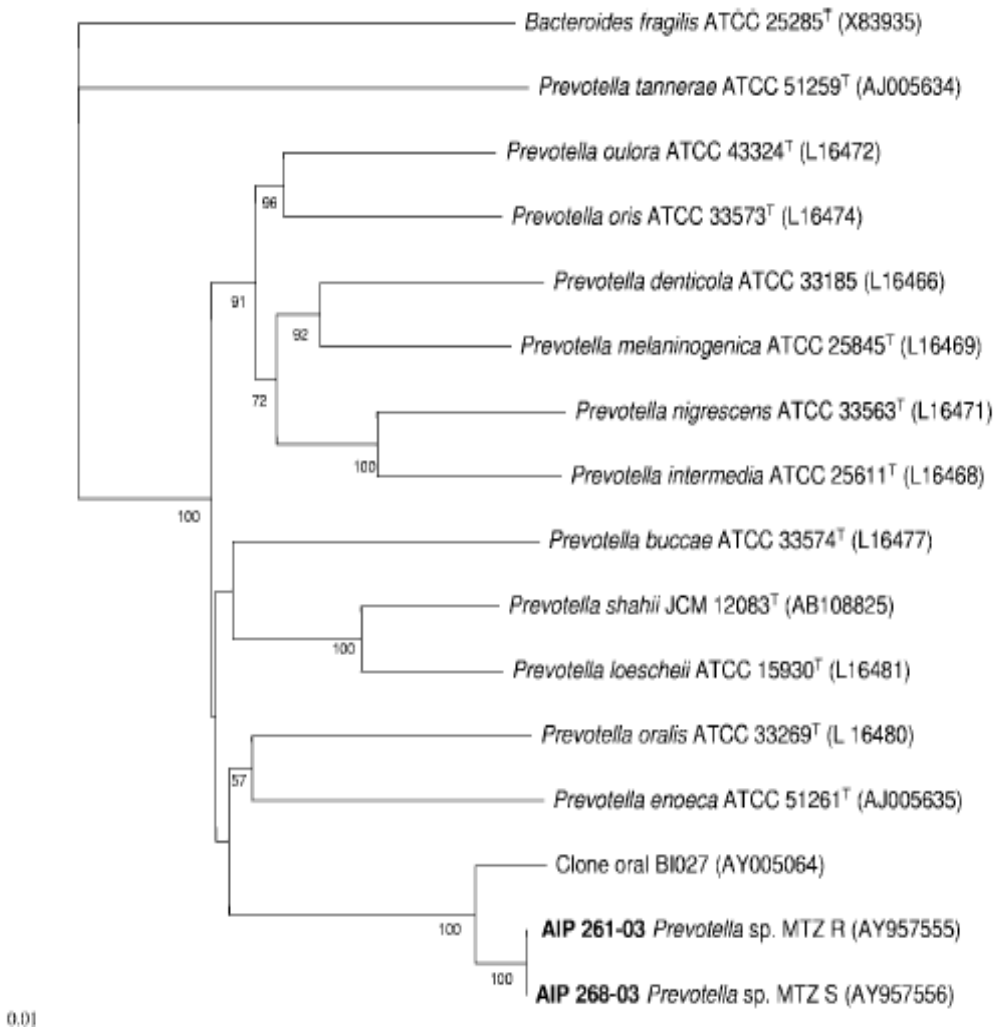

Figure 4: Phylogenetic position of *Prevotella* (Mory, Carlier et al. 2005)

An increasing number of *Prevotella* was found in children from rural Africa, who consumed high dietary fiber compared to children consuming a Western diet (De Filippo, Cavalieri et al. 2010).

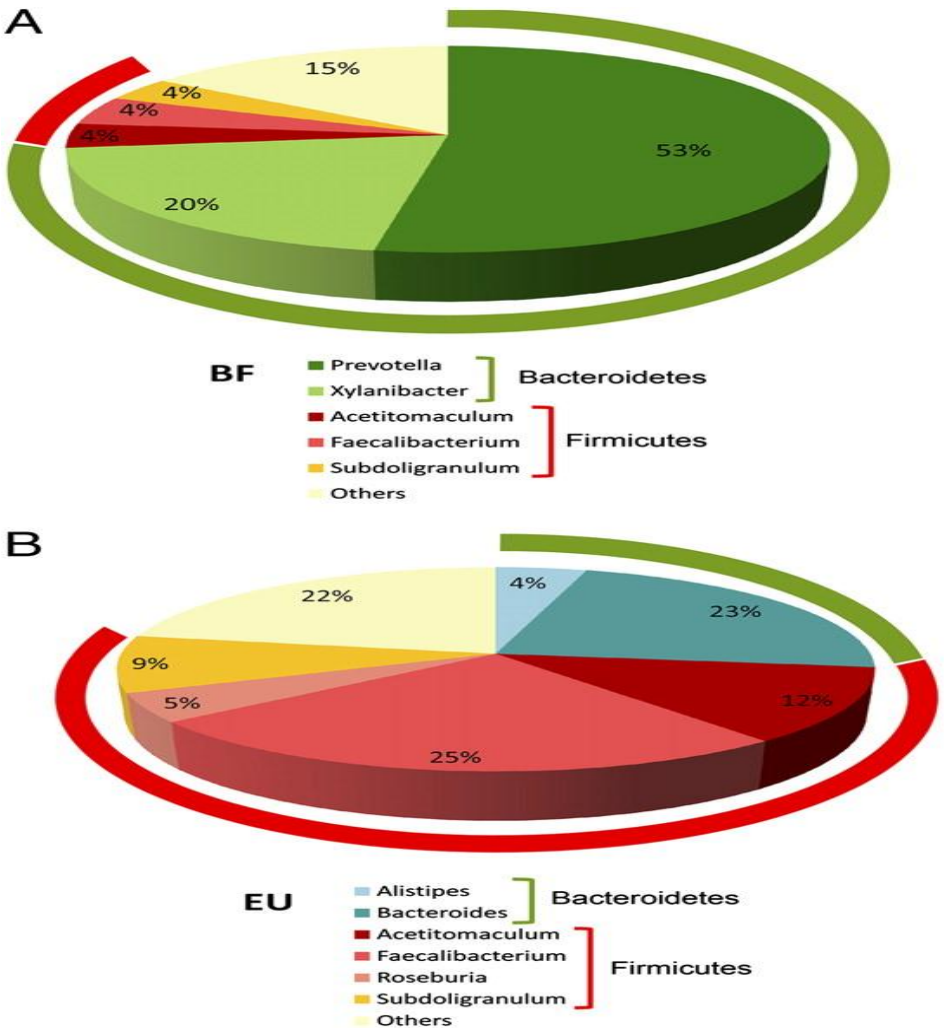

Figure 5: Comparison of healthy children from rural Africa (BF) and healthy European children (EU) (De Filippo, Cavalieri et al. 2010)

Another study in Malawi and Venezuela were plant food dominate, children and adults have a higher abundance of *Prevotella*. *Prevotella* is associated with carbo-hydrates and simple sugar consumption (Lozupone, Stombaugh et al. 2012).

7.4.2. *Akkermansia*

Domain	Bacteria
Phylum	Verrucomicrobia
Class	Verrucomicrobiae
Order	Verrucomicrobiales
Family	Verrucomircobiaceae
Genus	Akkermansia
Type species	Akkermansia muciniphila

Table 4: Classification of the genus *Akkermansia*

Akkermansia is a genus within the phylum *Verrucomicrobia*. *A. muciniphila* is a gram – negative anaerobe bacterium and is an essential part of the gut microbiota (Derrien, Vaughan et al. 2004).

The colonization of *A. muciniphila* starts in the early childhood, the density reaches about 3% of the total fecal bacterial count (van Passel, Kant et al. 2011).

Figure: The figure below shows the presence of *A. muciniphila* between differing age groups. There is a significant increase from the child to the adult. In early childhood, *A. muciniphila* colonize the intestinal gut and grow significant within the first year. The box plot shows an increase of this bacterium between a six month old child and their twelve month old counterpart. An opposite tendency is demonstrate in elderly. The availability of *A. muciniphila* decrease significant in older people compared to adults (Collado, Derrien et al. 2007).

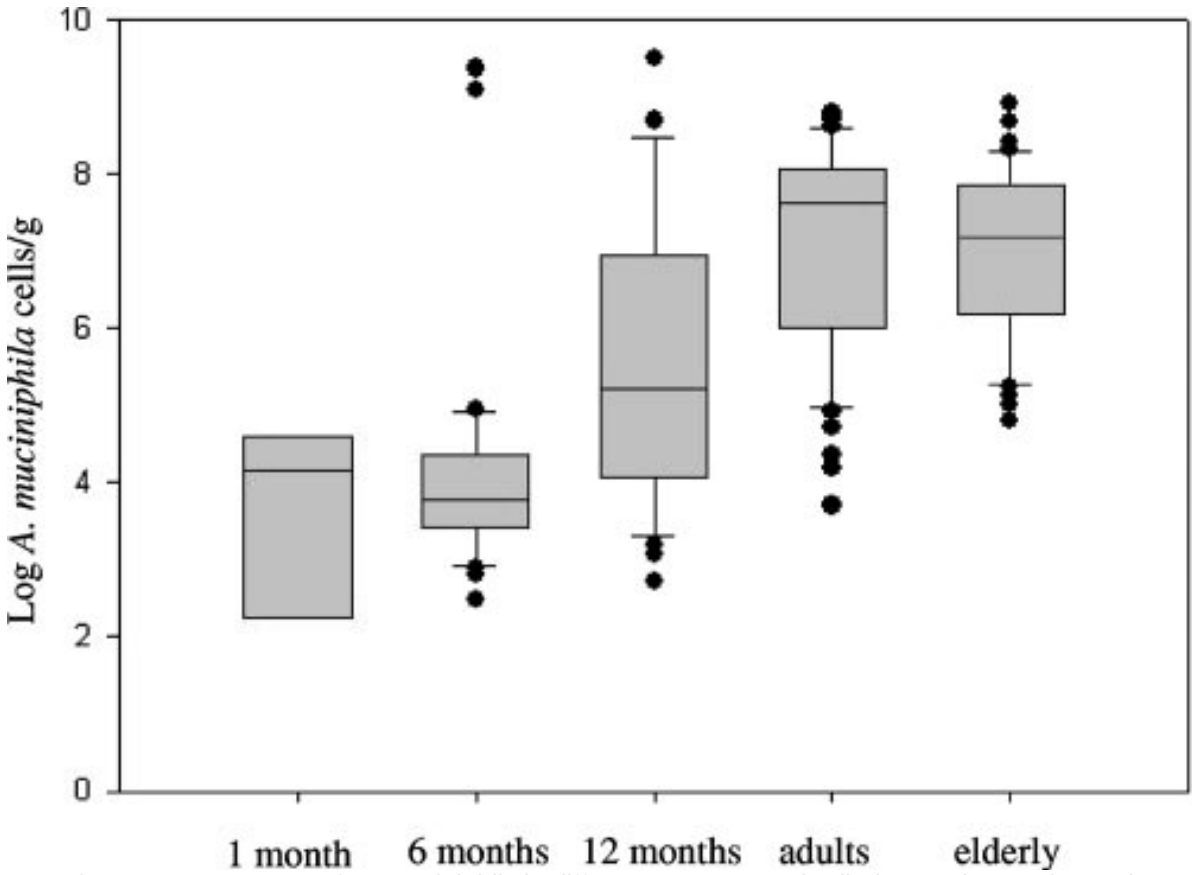

Figure 6: Occurrence of *A. muciniphila* in different age groups (Collado, Derrien et al. 2007)

The function of *A. muciniphila* is to decompose mucin, a glycoprotein that covers the intestine epithelium with a protective mucus layer (van Passel, Kant et al. 2011). It is capable of processing to benefit mucus as a carbohydrate- and nitrogen source (Belzer and de Vos, 2012). Mucin is the main element of the protective mucus layer of the human gut epithelium, in which bacteria live close to human cells.

A. muciniphila produces as result of mucus degradation Oligosaccharide and short chain fatty acids (SCFAs) (Derrien, Vaughan et al. 2004).

These products stimulate the microbiota interactions and the host – reactions. Oligosaccharide and Acetate activate the expansion and the metabolic activity of the bacteria, which colonize in the near of the mucus layer. This colonization may have a resistance against pathogens (Belzer and de Vos 2012). Mucus – degrading bacteria are increased in inflammatory bowel disease, which indicates, that there is an enhanced mucus production (Derrien, Van Baarlen et al. 2011).

7.4.3. *Faecalibacterium prausnitzii*

Domain	Bacteria
Phylum	Clostridia
Class	Clostridia
Order	Clostridiales
Family	Clostridiaceae
Genus	Feacalibacterium
Species	F. prausnitzii

Table 5: Classification of the genus *Faecalibacterium*

F. prausnitzii is one of the most frequent anaerobe bacteria, in 16S DNA based molecular analysis. In older literature, *F. prausnitzii* is named as *Fusobacterium* (Lopez-Siles, Khan et al. 2012).

Faecalibacterium is a butyrate producer (Tremaroli and Bäckhed 2012). Butyrate plays a key role during the maintenance of the human gut health. As an energy source for the gut mucus layer and as an important regulator for several functions gene expression, inflammation, differentiation as well as apoptosis in host cells are the main functions. In addition, there is an evidence, that butyrate production may play a relevant position in the bacterial energy metabolism. Butyrate also has a crucial impact on the progress and the gene expression in gut cells. It is assumed,

that it plays a protective role against colon cancer and colitis (Duncan, Holtrop et al. 2004).

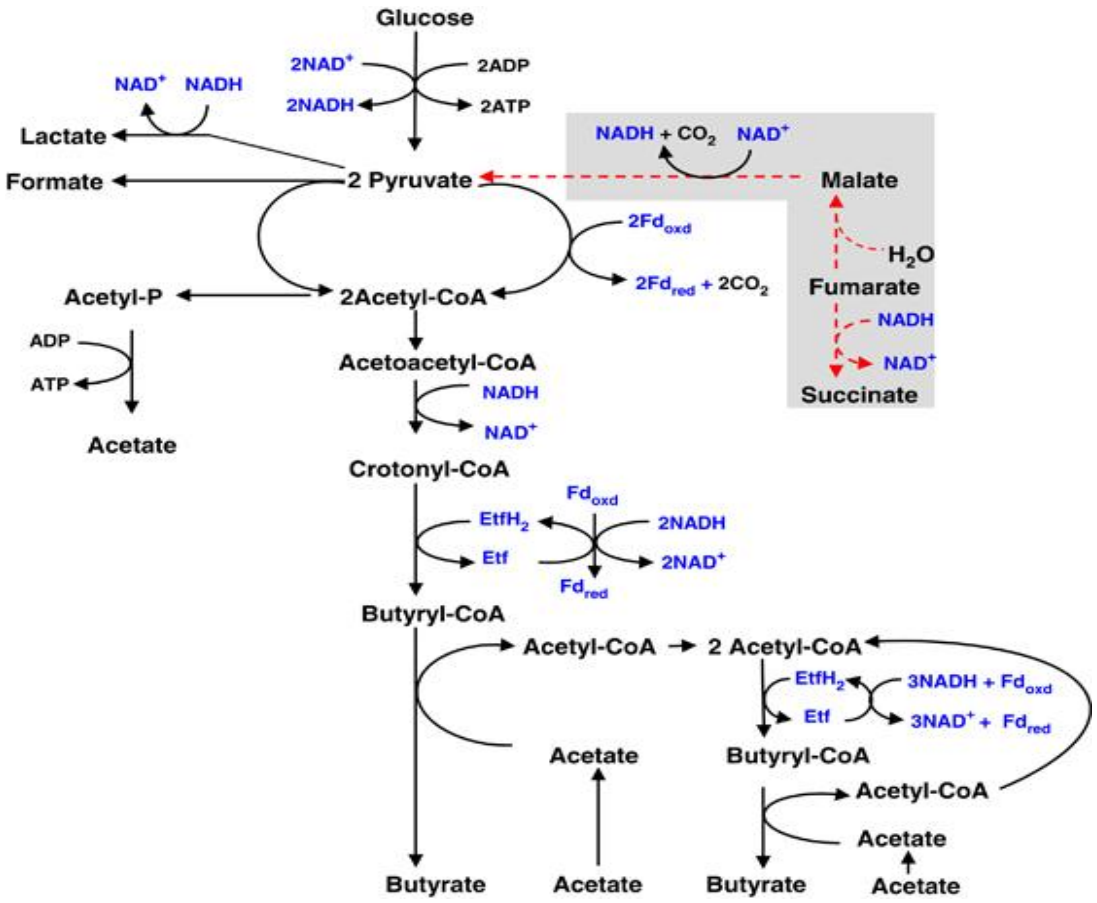

Figure 7: Production of butyrate and other short chain fatty acids of glucose by *F. prausnitzii* (Khan, Duncan et al. 2012)

F. prausnitzii has anti – inflammatory effects, partially, to block the nuclear factor – kappaB and also the secretion of pro – inflammatory mediators. The oral administration of *F. prausnitzii* increases the production of IL-10 by mono nuclear cells and reduces pro – inflammatory mediator like IL-12 in the colon. *F. prausnitzii* can play a role at the prevention of local gut inflammation and acute infection (Furet, Kong et al. 2010).

8. Aim of this study

Obesity is an increasing disease in our population and also a higher risk for concomitant diseases and complications Nutrition, sedentary lifestyle and missing exercise influence the body weight and the comorbidity.

New results of research indicate that the gut microbiota has an enhanced influence on the energy yield from the diet. A lot of animal studies indicate that obese mice and lean mice have different bacterial populations in their gut. So, the composition of the nutrients, the digestion, the microbial intestinal inhabitants and other factors play also an important role at the development of obesity. In obesity, not only the microbiota is different to lean people, also the intestinal barrier is disrupted.

This consequences are not only limited to an inflammation in the gut. Fat deposits, fatty liver and disturbed inulin sensitivity are the effects. Through these processes, a damaged gut microbiota influences the development of obesity, high blood pressure, lipid metabolism disorder and insulin resistance.

The aim of this study was to define differences in the feces of lean and obese/ type 2 diabetes people. This study focuses at three bacteria groups - *Prevotella*, *Akkermansia* and *Faecalibacterium*. The aim of this study is to find out the differences of gut microbiota in obese and type 2 diabetics.

We analyzed the effects of the 16S rRNA with real time PCR. We also compared the gut microbiota inhabitants between lean and obese/ type 2 diabetes participants.

9. Methods and Protocols

9.1. DNA-Extraction QIAamp® DNA Stool Mini Kit (50)

The DNA Extraction QIAamp® DNA Stool Mini Kit allows fast and easy purification of total DNA from fresh or frozen stool samples, so that they can used for PCR or other downstream enzymatic reactions or for storage at -20°C. The purified DNA is eluted in a low-salt buffer and is free of nucleases, protein and other source of irritations or inhibitors.

- routine procedure to collect DNA – following steps are important:

1. lysis of stool samples in Buffer ASL
2. absorption of impurities to inhibitEX matrix
3. purification of DNA on QIAmp Mini spin columns

- the purification of DNA can be used for PCR, other enzyme reactions or storage

Follow the steps according to the instruction of the DNA-Extraction QIAamp® DNA Stool Mini Kit (50) as listed below:

1. Measure the pH-value with the strips- wet the stripes with distilled water and dip the strip in the stool sample; then compare the strip with the pH- table.

2. Under using a spatula weight in 180 – 200mg froze stool sample a 2ml matrix E (soil kit) tube and supply with 1,4ml ASL buffer.

Pay attention, that the samples do not become liquid until ASL buffer is added and vortex immediately until the stool sample is homogenized to ensure maximum DNA concentration. ASL buffer provides the ability to remove inhibitory

substances from the stool samples.

3. Transmit the lysing matrix tubes in the beadbeater and beat for two cycles of 45 seconds with one intervening minute on ice. The beadbeater destroys over 90% of the cells via cell “cracking” action rather than high shear. Also the cell wall of gram-positiv bacteria gets damaged.

4. Heat the samples for 5 minutes at 95°C. Take care, that the tubes are open. If they are closed, they can “explode” because the temperature is high after the beadbeating. Heating also increases total DNA and helps to lyse gram- positive bacteria too.

5. Vortex for 15 seconds and centrifuge for 1 minute at 14.000rpm to pellet stool and lysing matrix.

6. Transfer 2ml of the supernatant into a new 1,5ml eppendorf tube and discard pellet. Small amounts of pellet do not affect the procedure.

7. Add a half inhibitEX tablet to each sample and vortex immediately until the tablet is completely dissolved. The tablet inhibits the enzymes. To allow the inhibitors to adsorb inhibitors and DNA-degrading substances incubate for 1 minute at room temperature to delete all source of irritation.

8. Centrifuge for 6 minutes at 14.000rpm to pellet the tablet with bound inhibitors.

9. Pipet the supernatant into a clean 1,5ml eppendorf tube and discard pellet.

10. Centrifuge for another 3 minutes at 14.000rpm.

11. Pipet 25µl proteinase K into a clean 2ml eppendorf tube.

12. Transfer approximately 600µl supernatant form step 10 to proteinase K.

13. Add 600µl buffer AL and vortex for 15 seconds. Never change the order of the steps 11,12,13.

14. Site the tubes at 70°C for 10 minutes. On the heating bloc.

15. Add 600µl ethanol (96 – 100%) to the lysate and vortex for mixing.

16. Label the lid of a new QIAamp spin column placed in a 2ml collection tube. Apply 600µl lysate from step 15 without moistening the rim. Centrifuge for 1 minute and discard the filtrate. Place the QIAamp spin column into a new collection tube and repeat the step until all lysate has been solved.

17. Add 500µl Buffer AW1 and centrifuge for 1minute at 14.000rpm. Discard the filtrate and put it into a new collection tube.

18. Add 500µl Buffer AW2 and centrifuge for 3 minutes at 14.000rpm. Discard the filtrate and place it into a new collection tube.

19. Centrifuge for another 1 minute to remove residual buffer AW2.

20. Place the QIAamp spin column into a new 1,5ml eppendorf tube and elute bounded DNA with 200µl DNAse and RNAse free water (preheated to 80°C).

21. Centrifuge for 1 minute. This sample is the UR- solution. Pipette 20µl of the UR- solution in a 1,5ml eppendorf tube and label it with “Aliquot”.

22. Repeat the step 20 and 21 for the first elution. Pipette again 200µl preheated DNAse and RNAse free water and centrifuge for 1 minute.

23. Measure the DNA concentration and the purity by Picodrop (ng/µl)

24. For storage keep the eluates at -20°C.

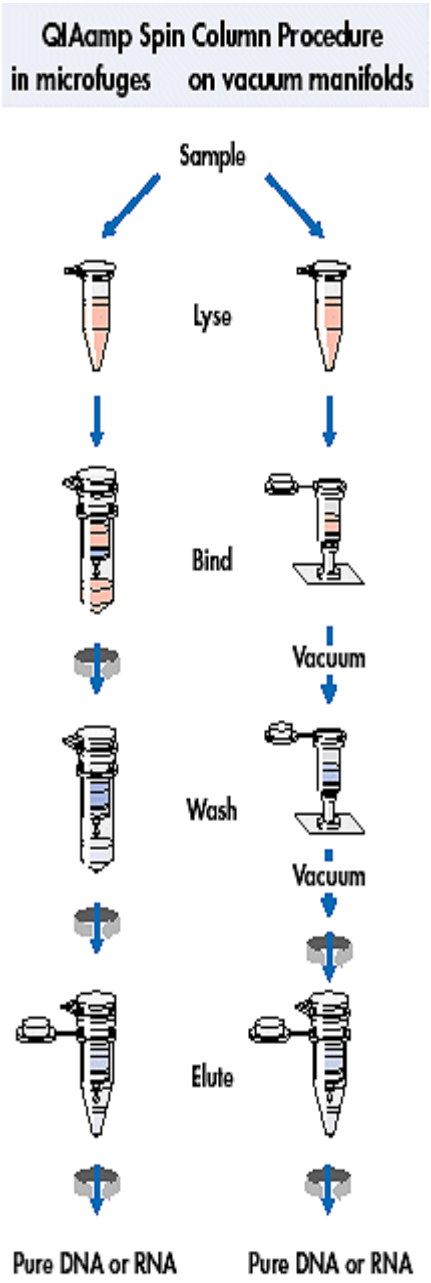

Figure 8: QIAamp DNA Stool Mini Procedure (QIAGEN, 2010)

9.2. Real time Polymerase chain reaction

The real time PCR or quantitative PCR is a multiplication method for nucleic acids. The main principle is that a defined sequence segment is isolated with a specific oligonucleotide (Primer). The application occurs with a heat – resistant DNA – Polymerase.

The principle PCR – steps:

1. *Denaturation* – the temperature raise up to about 94°C and both strains of the template DNA unravel.
2. *Annealing* – this step is important for the Primer to bind/ hydride on the DNA. The temperature reduces.
3. *Elongation* – the temperature increases to the optimum of the polymerase to allow a good second synthesis strand.
4. *Final Elongation* – this step make sure, that the polymerase finish the work.

This PCR conditions permit an enrichment of the sequence part. For each Primer pair, the terms of the PCR reaction have to be optimized. Variable steps, as kind of Taq – Polymerase, SYBR – Green, concentration of Magnesium, number of cycles and the temperature profile of the PCR – program has to be adapted.

Melting temperature provides information on the binding to the template. It shows the temperature, in which 50% of the primer does not bind to the template.
A distinction is possible, because the double – stranded DNA of a specific PCR product has a higher melting point than the resulting non – specifically primer. The amount of the peak gives information about the intensity of the formed fragment.

A standard curve is important for the quantification to compare the samples. The concentration of the standard curve has to be compared to the cycle threshold value of each run. A standard, which is included in each PCR – run, helps to match the results.

A positive control is also integrated in every trial, because this kind of sample gives information if the pipetting was correctly and the reaction generally worked. Also a very essential sample is the negative control, which gives information about the cleanliness during the preparation.

The concentration from the DNA was measured by Picodrop. The spectrometer analyzes the sample. Measuring zones are:

- 260/280 protein
- 260/230 Phenol

The purity analyze is the quotient between the absorption 260nm and 280nm. The valuation under the value of 1,8, there is a contamination of the DNA with protein and phenol.

9.3. qPCR – program + Primer

Rotor Gene 3000 (Corbett Life Science) is the instrument for the qPCR in the lab. For all three types of bacteria SYBR GREEN (SensiMix™ SYBR No-ROX Kit (SYBR® Green) (3 mM $MgCl_2$) was used for measuring. All samples are tested in duplicate. Primer concentration was 50pmol/µl and the concentration of the samples 5ng/µl. For each bacterium a standard was prepared (see Appendix: cloning). Positive and negative control is included in every qPCR run.

Pipette schema:

components	x number of the samples
Sybr Green Master Mix	5µl
Primer forward	1µl
Primer reverse	1µl
NFW	1µl

MM'

Table 6: Pipette schema for qPCR

total volume: 8µl MM' + 2µl template* = 10µl

*Template is the topic for positive control, sample, negative control and standard.

9.3.1. *Prevotella*

standard: 3OH1 clone number 2

positive control: 2OH1

negative control: NFW

		Temperature	Duration	Repeat
initial Denaturation		95°C	10min	
cycling	Denaturation	95°C	15sec	} x 45
	Annealing	66°C	1min	
	Elongation	72°C	25sec	
final Elongation		72°C	1min	

Table 7: qPCR program for *Prevotella*

melt: 72°C – 95°C

10sec / 0,5°C

0,5°C/ cycle

primer	**sequence (5' – 3')**	**size (bp)**	**reference**
Prev F	CACCAAGGCGACGATCA	283	(Larsen, Vogensen et al. 2010)
Prev R	GGATAACGCCYGGACCT		

Table 8: Primer for *Prevotella*

9.3.2. *Akkermansia*

standard: 2OC2 clone number 1

positive control: 2OH1

negative control: NFW

		Temperature	Duration	Repeat
initial Denaturation		95°C	5min	
cycling	Denaturation	95°C	15sec	} x 40
	Annealing	60°C	40sec	
	Elongation	72°C	30sec	
final Elongation		72°C	5min	

Table 9: qPCR program for *Akkermansia*

melt: 72°C – 95°C

10sec / 0,5°C

0,5°C / cycle

primer	**sequence (5' – 3')**	**size (bp)**	**reference**
AM 1	CAGCACGTGAAGGTGGGGAC	327	(Collado, Derrien et al. 2007)
AM 2	CCTTGCGGTTGGCTTCAGAT		

Table 10: Primer for *Akkermansia*

9.3.3. *F. prausnitzii*

standard: 1OH2 clone number 2

positive control: 4OH1

negative control: NFW

		Temperature	Duration	Repeat
initial Denaturation		95°C	10min	
cycling	Denaturation	95°C	15sec	} x 40
	Annealing	62°C	40sec	
	Elongation	72°C	30sec	
final Elongation		72°C	5min	

Table 11: qPCR program for *F. prausnitzii*

melt: 72°C – 95°C

10sec / 0,5°C

0,5°C / cycle

primer	**sequence (5' – 3')**	**size (bp)**	**reference**
FPR 2F	GGAGGAAGAAGGTCTTCGG	248	(Ramirez-Farias, Slezak et al. 2009)
Fprau 645R	AATTCCGCCTACCTCTGCACT		

Table 12: Primer for *F. prausnitzii*

10. Results and Discussion

All methods, used for determination, show a high variability of microbiota. We compared the samples of the same individual over the three time points as well as between subjects. The variation between the different patients is higher than in the samples of the same person. The diversity indicated also a hierarchy of the groups. Type 2 diabetics showed a lower diversity than the obese patients and in the lean control group the variety showed the highest bacterial composition.

Obesity and type 2 diabetics are associated with changes in the composition of the gut microbiota. Also the diversity and abundance altered compared to the healthy lean counterparts. Microbiota interacts with different functions like body weight, nutrition intake, inflammation, energy extraction as well as gut barrier.

In my master thesis, I focused on the three bacteria groups:
Akkermansia, *Prevotella* and *Faecalibacterium prausnitzii*.

Akkermansia increased in type 2 diabetics during the study period. The abundance was already higher in this group compared to obesity and lean controls. A*kkermanisa* are not depending on the food intake by the host and this enhanced frequency in type 2 diabetes patients may reflect a better setting to caloric restriction compared to the rest of the host, which don't depend on the host nutrition.

Prevotella play an important function in the gut because of their role in the development of the discussed enterotypes. We noticed no significant difference of *Prevotella* between the three groups or time points. We also recognized no deviation between type 2 diabetes persons during the study period, even though the patients lost weight. The HbA1c blood levels are also well adjusted in type 2 diabetics. The figure nine below shows the different three groups over the time points.

There are now significant difference between lean control (LC), obese control (OC) and diabetics (D).

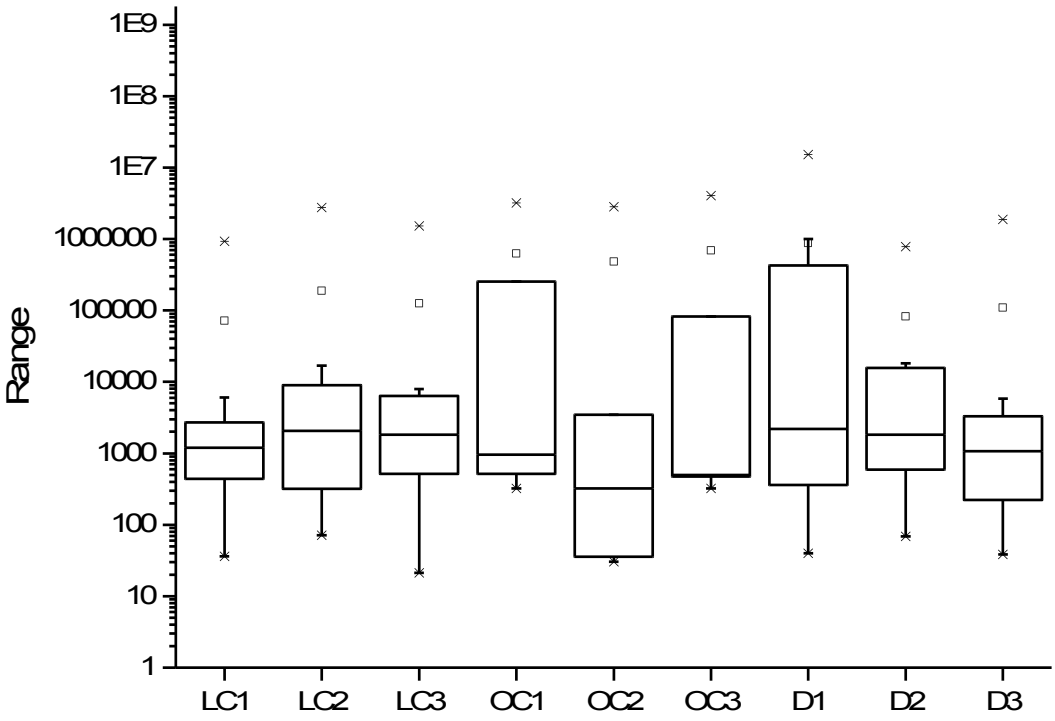

Figure 9: Origin - *Prevotella*, unpublished data

For *Faecalibacterium prausnitzii*, we observed a decrease in type 2 diabetics and obese patients. These two groups differ significantly over the three time points compared to the lean controls with less *F. prausnitzii*. Butyrate producers, like *F. prausnitzii*, may have an influence in developing obesity.

11. Papers

11.1. Abundance and diversity of microbiota in type 2 diabetes and obesity

Remely Marlene, Dworzak Simone, Hippe Berit, Zwielehner Jutta, Aumueller Eva, Brath Helmut, Haslberger Alexander

Paper: Journal of Diabetes & Metabolism, accepted manuscript, 2013

Abstract

Inflammatory reactions leading to the progression of metabolic syndrome contribute to changes in composition of the GIT microbiota.

We characterize fecal microbiota at three time points in fourteen obese participants, nineteen lean controls and twenty-four type 2 diabetes patients. Obese and type 2 diabetics received an intervention of nutritional counseling, type 2 diabetics an additional therapy with a GLP-1-Agonist. The microbiota composition was analyzed for abundance and diversity by quantitative real-time polymerase chain reaction, denaturing gradient gel electrophoresis and high throughput sequencing.

In type 2 diabetics an increase of diversity was observed with intervention whereas the values of lean controls remained unaffected. In the lean and obesity groups, a lower *Firmicutes: Bacteroidetes* ratio correlated with lower BMI. In type 2 diabetics the ratio of *Firmicutes* to *Bacteroidetes* increased throughout the intervention period. Type 2 diabetics showed a significantly enhanced proportion of lactic acid bacteria before and after intervention, also *Akkermansia* and *Enterobacteria* showed a higher abundance in type 2 diabetics, increasing throughout the study period. *Archaea* were significantly more frequent in type 2 diabetics.

Whether differences seen in abundance of certain groups and diversity of microbiota reflect different underlying inflammatory mechanisms of type 2 diabetes and obesity or rather the progression of the metabolic disease remains unclear and needs long-term investigation.

Keywords

GLP-1-Agonist; metabolic syndrome; ratio *Firmicutes: Bacteroidetes*; lactic acid bacteria;

Akkermansia; *Prevotella*; Enterotypes

Abbreviations

ANOVA- analysis of variance

AMPK - AMP-activated protein kinase

BMI – body mass index

DGGE – denaturing gradient gel electrophoresis

DSM – Deutsche Stammsammlung von Mikroorganismen

FFG – Austrian Förderungsgesellschaft

FFQ – food frequency questionnaire

FIAF - fasting-induced adipose factor (or angiopoietin-like protein 4)

GI – gastro intestinal

GLP – glucagon-like peptide

Gpr – G-protein coupled receptor

HbA1c – glycol hemoglobin

IL– interleukin

LAB – lactic acid bacteria

LPS - lipopolysaccharide

MCP-1 – monocyte chemo attractant protein-1

P – probability

PAI-1 - plasminogen activator inhibitor-1

PC – principal component

PCA – principal component analysis

PCR – polymerase chain reaction

PYY – Peptid YY

qPCR – quantitative polymerase chain reaction

rDNA – ribosomal DNA
SCFA – short chain fatty acid
T – time point
TLR – toll like receptor
TNFα – tumor necrosis factor alpha
UPGMA - unweighted pair group method with arithmetic mean
UK – United Kingdom

Introduction

The gut microbiota is an integral part of a complex network coordinating the physical and chemical elements of the intestinal barrier together with the immune, sensory, neuromotor- and enteroendocrine systems [1]. It bridges the indeterminate gap between food and weight by affecting the host metabolism through regulation of intestinal glucose absorption, lipogenesis and fat deposition.

Housekeeping functions such as the central carbon metabolism or the amino acid synthesis of important protein complexes are present in every bacterium. Secondary metabolisms are related to a minimal metagenome, for example the biodegradation of complex sugars and glycans [2]. Profiles of human gut microbiota reflect the metabolic cooperation between different phylotypes since no genus can degrade all substrates; for example establishing all short chain fatty acids (SCFAs). SCFAs have a role in the regulation of energy metabolism, immunity and adipose tissue expansion [3]. Gut microbiota of all human beings fulfill core functions, but differ in specialist functions, as a consequence some communities are linked to human diseases and obesity more than others [2].

Observations of experiments with germ-free mice compared to conventional raised mice showed independent from diet a weight gain according to gut microbiota [4]. Studies on the relationship between gut microbiota composition and obesity show an increased number of *Firmicutes* and a reduction of *Bacteroidetes* in obese mice and humans compared to lean controls [4-7]. A controlled diet and weight-loss re-

versed the observed ratio of *Firmicutes* to *Bacteroidetes* [4, 8-10]. The number of *Bacteroidetes* depends on the weight loss rather than on caloric intake [7, 11] whereas the *Firmicutes* group remains unchanged and no correlation with total caloric intake can be detected [10]. The ratio reaches a lean type profile after a one-year period of diet-induced weight loss [12]. The abundance of *Firmicutes* in diabetic persons is significantly lower compared to non-diabetics, while *Bacteroidetes* and *Proteobacteria* are more abundant in diabetics.

The ratio of *Bacteroidetes* to *Firmicutes* is significantly and positively correlated with reduced glucose tolerance, but negatively correlated with higher BMI. Especially the relative abundance of *Bacilli* is increased in diabetic mice models and humans. Relative abundances of *Actinobacteria* and *Verrumicrobia* do not differ between diabetic and non-diabetic individuals. *Bacteroides-Prevotella* versus *C. coccoides - E. rectale* groups are positively associated with plasma glucose, but negatively correlated with BMI [13]. Wu et al. (2009) found a higher abundance of *Bacteroides* (frequency of 53,6%), and lower abundance of genus *Prevotella* (10,7 %), genus *Proteobacteria* (3,6 %) and phylum *Firmicutes* (10,8 %) compared to healthy volunteers in sequence analysis of selected DGGE amplicons, whereas qPCR results indicate a lower copy number of *Bacteroides vulgatus* and *Bifidobacterium* in the fecal microbiota. The *Clostridium leptum* cluster was more abundant in the diabetic than in the healthy group [4, 11, 14]. It is estimated that subjects with type 2 diabetes are relatively enriched with Gram-negative bacteria, belonging to the phyla *Bacteroidetes* and *Proteobacteria*, which can elicit endotoxemia [13] through increased plasma LPS [11, 13, 15-18].

Obese individuals may have a microbiota more efficient in extracting energy and may be enriched in enzymes for the breakdown of otherwise indigestible alimentary polysaccharides and in other transport proteins and fermentation enzymes and further induce insulin resistance and diabetes [4]. Metabolic endotoxemia signifi-

cantly correlates with oxidative stress, macrophage infiltration markers and all inflammatory markers triggering insulin resistance (PAI-1, IL-1, IL-6 and TNF-α) [4, 10, 15, 19]. In a high fat diet elevated LPS increase gut permeability, by affecting GLP-1, GLP-2 and PYY [10, 11]. An absence of LPS receptor CD14 reduces the high-fat diet induced effects on adipose tissue inflammation, blood glucose profiles, insulin resistance index, glucose-induced insulin secretion [7, 10, 15], hepatic steatosis, liver inflammation and adipose tissue macrophages infiltration [18]. Metabolic endotoxemia is indicated by a change in the intestinal microbiota as antibiotic treatment reduces plasma LPS levels [7, 10, 11, 20]. An early modification of the microbiota with probiotics or prebiotics might be a beneficial influence [11].

The impact of the GLP-1-Agonist Liraglutide on blood glucose level, weight management and systolic blood pressure was conducted e.g. in the LEAD (Liraglutide Effect and Action in Diabetes) and in the DURATION study programs. GLP-1-Agonists lead to a glucose-dependent increase of insulin secretion in beta cells and suppress the glucagon secretion in pancreatic alpha cells. Additionally, it slows the gut passage and therefore decreases the postprandial spikes in blood glucose. Furthermore, GLP-1-Agonists decrease body weight, Liraglutid showed in one study that 25 % of patients lost significant weight, on average 7.7 kg [21].

The objective of this study was to investigate differences of gut microbiota in obese and type 2 diabetics, and the response to intervention for estimating its potential role in controlling metabolic syndrome.

Materials and methods

Ethics statement

The project “Abundance and diversity of microbiota in type 2 diabetes and obesity” was approved by the Viennese Human Ethics committee. We received a signed form of consent from all study participants for using stool samples and data obtained from food frequency questionnaires for the analyses presented here.

Study participants and study design

Twenty-four insulin-dependent type 2 diabetes patients (aged 56 +/- 9) were enrolled in cooperation with a Diabetes Oupatient Clinic. In addition we enrolled fourteen obese participants with no established insulin resistance (aged 38 +/- 14) and nineteen healthy lean controls (aged 30 +/- 8) (Table 1). Obese and type 2 diabetics received an intervention of nutritional counseling, type 2 diabetics an additional therapy with GLP-1-Agonists (Liraglutid). To ensure comparable data, patients were interviewed for their history of gastrointestinal diseases, use of antibiotics, probiotics or prebiotics. BMI, age and lifestyle habits were assessed in combination with a retrospective food frequency questionnaire.

Fecal samples were collected at three occasions; before treatment, during and after treatment, with a distance of 1 month between the first two time points and 3 months between the second and third time point. Controls also donated three fecal samples over the course of four months.

Stool sample processing and extraction

Stool samples were stored immediately after collection at -18 °C in subject´s home freezer and at - 70 °C upon arrival in the laboratory.

Total bacterial DNA was extracted from fecal samples using the QIAamp® DNA Stool mini kit (QIAGEN, GmbH, Germany) according to the manufacturer´s protocol. Additional samples were treated in FastPrep™ Lysing Matrix E tubes twice for

45 sec in a bead-beater (Mini-Beadbeater-8) with an intervening minute on ice. DNA concentration and quality was determined by picodrop (Picodrop, UK) and agarose gel-electrophoresis.

Clone library

For further analysis in qPCR we created clone libraries from dominant members of *Akkermansia muciniphila, Prevotella intermedia and Lactobacillus casei* in two stool samples of healthy volunteers.

PCR-products amplified with group specific primers (table 2) where inserted into p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Clone libraries were screened according to Schabereiter-Gurtner et al., 2001 [22]. The obtained nucleotide sequences from Sanger sequencing were corrected for vector sequences and taxonomically identified using the ribosomal database project 10 (http://rdp.cme.msu.edu/).

Real-time qPCR

Bacterial abundance was quantified by qPCR using TaqMan qPCR and SYBR Green qPCR in a Rotorgene 3000 (Corbett Life Science) using 16S rDNA group specific primers (Tables 2-3). Specificity was checked with the ProbeMatch function of the ribosomal database project 10 (http://rdp.cme.msu.edu/). The PCR reaction mixture and serial DNA dilution of typically strains were prepared according to Pirker et al. 2012 [23].

PCR/DGGE

The diversity of total bacteria was measured by DGGE using the primer set 341f-GC 5'-CCT ACG GGA GGC AGC AG-3' [24] and 518r 5'-ATT ACC GCG GCT GCT GG-3' [25]. The endpoint PCR was carried out with a ready-to-use GoTaq® Green Master Mix (Promega) with 1.5 mM MgCl2 in a 96-well Gradient Thermal Cycler (Labnet MultiGene™).

DGGE gels were prepared as described previously [26] with a linear gradient of 25–65 % for total bacteria using a peristaltic pump. The reference marker contained 16S rDNA gene fragments of *Lactobacillus reuteri DSM 20016, Escherichia coli 1029, Enterococcus faecium DSM 20477, Clostridium blautia coccoides DSM 935, Clostridium leptum DSM 753, Bifdobacterium longum sp. longum DSM 20219* and *Bacteriodes theataiomicron DSM 2079.*

High throughput sequencing

Sixteen samples were analyzed with Roche GS FLX+ Titanium next generation sequencing (LGC sequencing GmbH, Berlin). For alignment and further analyses we used the pyro pipeline of the ribosomal database project 10 (http://rdp.cme.mus.edu/) and qiime. We then performed significance UPGMA clustering, and principal coordinate analysis in qiime. We used jackknifing to determine results.

Statistical analysis

We used the non-parametric Mann-Whitney U test and for three unpaired groups the non-parametric Kruskal Wallis ANOVA for comparison of the non-parametric unpaired values in OriginPro version 8 (OriginLab, Northampton, MA). P values < 0,05 were determined as statistically significant. DGGEgels were evaluated with Gel compare II (Applied Maths).

Results

All used methods indicate high individual variability. However the variation within samples of the same individual over time was consistently lower than variation between subjects. Our approach was to first compare the abundance of each bacterial group individually, then to calculate the *Firmicutes* to *Bacteroidetes* ratio and finally to compare our results with discussed enterotypes. We applied PCRDGGE

fingerprinting to estimate the species richness of all bacteria and selected sixteen samples for 454 sequencing to obtain in-depth data on microbial diversity.

Analyses of quantitative GI microbiota composition

We did not detect any differences between the three groups in total bacterial abundance. However, the microbial composition showed significant differences between type 2 diabetics, lean controls and obese participants. The most abundant bacterial groups in lean, obese and type 2 diabetics were the Gram-positive bacteria belonging to the *Clostridium Cluster IV* and the *Clostridium Cluster XIVa* as well as the Gram-negative *Bacteroidetes*, which altogether made up a median proportion of more than 95 % of all detected bacteria. The ratio of *Firmicutes: Bacteroidetes* increased in type 2 diabetics under dietary intervention and therapy with GLP-1-Agonist during the study period (T3 $p = 0.04$) from an already higher *Firmicutes: Bacteroidetes* ratio at the first time point ($p = 0.02$). In the obese and lean groups, a lower *Firmicutes: Bacteroidetes* ratio correlated with lower BMI. *Clostridium* Cluster *IV* and *Clostridium* Cluster *XIVa* abundances were not significantly different between the groups or time points, but we observed a decrease in intervention of type 2 diabetics. Lactobacilli were significantly more abundant in type 2 diabetics at baseline ($p=0.01$) and increased over the study period (T3 $p = 0.004$; figure 1), compared to the other groups. High throughput sequencing confirmed these findings.

Overall type 2 diabetics were enriched in *Firmicutes* especially *Clostridiales* and *Bacilli*, especially *Lactobacillales*. In accordance *Bacteroidetes* were less abundant in diabetics, resulting in a higher *Firmicutes: Bacteroidetes* ratio. Between the first and third time point *Bacteroidetes* decreased and *Firmicutes* increased. Sequencing and qPCR were congruent in finding a *Firmicutes: Bacteroidetes* ratio of 1 in lean controls (figures 2 and 3). No significant changes could be detected within the *butyryl- CoA: acetate CoA-transferase* gene abundance between the study groups,

this butyrate production gene tended to be more abundant in type 2 diabetics. *Archaea* as well as species of the genera bifidobacteria, enterobacteria*, Akkermansia and Prevotella* accounted for the remaining proportion of the microbiota. Enterobacteria were more abundant in type 2 diabetics at all time points compared to the control group. This observation was significant at the first two time points despite greater variability in diabetics (T1 p = 0.04; T2 p = 0.01). Greater abundance of enterobacteria was also detected in obese study participants. We observed no significant differences of *Prevotella* and bifidobacteria between the three groups or time points. *Akkermansia* increased throughout the study period in type 2 diabetics, with an already higher initial abundance compared to lean controls and obese subjects in data obtained from qPCR as well as in 454 sequencing results (p = 0.16).

At all three time points *Archaea* were significantly (p = 0.05) more frequent in the type 2 diabetics (18 ± 5) compared to control groups, followed by obese patients with 4 (± 0) and lean controls (5 ± 3).

Diversity analyses

DGGE fingerprinting indicated highly diverse individual differences of the microbiota. The dataset was subjected to principal component analysis (PCA). The first two principal components (PCs) explained 15.6 % and 12.0 % of variance (data not shown). Although overlapping, PCA showed grouping of band patterns according to the three groups of study participants. Between the groups a ranking of diversity was observed, as type 2 diabetes patients had the lowest diversity, followed by obese participants and the highest diversity in the lean control group. Differences between type 2 diabetics and lean controls were significant at time point 2 (p = 0.03) and time point 3 (p = 0.01) (Table 4).

Shannon's diversity index of 454 sequencing results showed no significant differences between the three groups (D 5.19 +/- 2-62; LC 6.48 +/- 0.47; OC 4.51 +/-

2.60). Lean controls had a higher Shannon index and a higher number of sequences (683.3 +/- 59.6) compared to obese controls (571.5 +/- 48.2) and type 2 diabetics (T1 645.5 +/- 22.6; T3 619.0 +/- 62.3). PCA was also calculated on the 454 sequencing data. The first three PCs explained 44.6 %, 17.2 % and 8.3 % of the variance in the data. Samples of the diabetic group, obese group and the control group at phylum level were clustered along PC 1 and PC 2 (figure 4). In the PCA of sequencing results, samples of one participant at the two different time points and also the three different groups were grouped according to their variance along principal components. The first PCA was attributed to *Firmicutes* and *Verrumicrobia,* it contributed most to the separation along PC1 and *Proteobacteria, Actinobacteria* and *Bacteroides* contributed to the clustering along PC2. PCA of multiple genera showed grouping of the subjects with diabetes (figure 4). Additionally Jackknifing did not affect the UPGMA unifrac clustering results (figure 5).

Food Frequency Questionnaire (FFQ)

Analysis of the participant´s FFQ did not reveal any differences in the consumption of grain, vegetables, fruits, meat, dietary products and fish. Mostly type 2 diabetics consumed sweets less than once a week (40.0 % T1, 28.0 % T2) in contrast 28.0 % of lean individuals consumed sweets three to five times per week and 50.0 % of obese controls consumed sweets one to three times per week.

However, study participants stated an average consumption of sweets 1-15 times per week. The main differences are seen in life style patterns due to the lower physical activity in obese (37.5 % T1, 12.5% T3 never do sports) and type 2 diabetics (64.0 % T1, 52.0 % T3 never do sports), whereas 44.0 % of lean controls did sports 1-3 times per week. HbA1C blood level was determined at laboratory Health Center South in Vienna. The levels were between 7-8 % in our type 2 diabetes group. 19 out of 24 type 2 diabetes patients showed an average of 3.5 % (± 1.9) of weight reduction.

Discussion

This study focused on the characteristics of fecal microbiota in type 2 diabetic patients, before and after intervention with nutritional counseling and therapy with GLP-1-Agonist, comparing to a group of obese volunteers receiving nutritional counseling and a lean group.

Similar counts of total bacterial abundance

In this study we did not observe any significant differences in total bacterial abundance between type 2 diabetes, obese and lean controls, as a possible result of the intervention. Accordingly Larsen et al detected similar counts of total bacterial abundance in type 2 diabetics and the control group [13].

Bifidobacteria showed no significant differences between the three groups and intervention, possibly reflecting no significant differences in food composition or as an indicator for the well-adjusted HbA1c values. Wu et al 2010 reports a reduced abundance of bifidobacteria in type 2 diabetics in qPCR as well as in sequencing results as a possible characteristic of diabetes [12]. In mice a significant negative correlation has been observed between endotoxemia and *Bifidobacterium spp.*, but no relationship with any other bacterial group during treatment with high fat diet and oligofructose [27]. A treatment with prebiotics may improve or prevent disruption of intestinal epithelial barrier function, reduce intestinal permeability and consequently improve endotoxemia [20].

Moderate increase of diversity in type 2 diabetics with intervention

More than the abundance of total bacteria or certain bacterial groups the diversity of microbiota indicated by richness of species or gene counts is now seen as an important indicator of microbiota resilience and gut health [28]. Shannon's diversity index showed no significant differences between the groups, whereas band pattern analysis showed significant differences between the lean control group and type 2 diabetics. In type 2 diabetics an increase of bands was seen with intervention

whereas the values of lean controls remained unaffected (table 4). Wu et al., 2010 does not observe significant differences by comparing diversity profiles of type 2 diabetics and non-diabetics, which indicates an association of health and disease with a shift in the balance of gut microbiota rather than an action of a single microbe affecting the diversity [12]. Ley et al., 2005 observes a lower diversity in ob/ob animals with lower abundance of *Bacteroidetes* and a proportional increase in *Firmicutes* compared to lean mice. In this model the diversity is affected by obesity [29], however we indicated a lower diversity in type 2 diabetics, but not in obese volunteers. In agreement to our observed PCs, PC1 *Firmicutes* and *Verrumicrobia*, PC2 *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* (figure 4), Larsen et al., 2010 shows also a higher separation between the groups attributed to *Bacteroides*, *Firmicutes* and *Verrumicrobia* (45 %) and in second direction the combination of *Proteobacteria* and *Actinobacteria* (28 %) [13]. More than changes in the abundance of some microbiota groups or the small changes in body weight in some patients this increase in diversity could indicate the success of the nutritional/ GLP-1-Agonist intervention.

Higher ratio of *Firmicutes* versus *Bacteroidetes* in type 2 diabetes patients

We detected a higher abundance of *Firmicutes* in type 2 diabetics. *Firmicutes* (60 % at T3) increased and *Bacteroidetes* (35 % at T3;) decreased during intervention. The phylum *Fimicutes* harbours many butyrate and acetat producers. According to previous studies with higher concentration of fermentation end products in tissue and lower energy content in feces, *butyryl-CoA: acetate CoA-transferase* genes tended to be more abundant in type 2 diabetics, but this difference was not significant. Obese patients on the contrary showed a decreasing ratio of *Firmicutes* (55 %) to *Bacteroidetes* (45 %). Intervention here resulted in a weight loss of (4,87 ± 3,68 kg). Weight loss was highly diverse between individuals (figure 2,3).

Consequences of these shifts remain under discussion for their effects on endotoxin mediated low grade inflammation, but also for consequences on energy extrac-

tion and body weight. An increased *Firmicutes* to *Bacteroidetes* ratio is previously associated with increased body weight [6], but also controversial data exist, e.g. by higher Clostridia in association with higher plasma glucose [13]. A higher ratio of *Firmicutes* versus *Bacteroidetes* increases the energy harvest and the content of SCFA and as such the lipogenesis and accumulation in adipocytes [11]. Schwiertz et al. on the contrary reports a lower *Firmicutes* to *Bacteroidetes* ratio in overweight human adults compared to lean controls [8] whereas other working groups do not find an association between the *Bacteroidetes* to *Firmicutes* ratio and BMI [9, 20, 30, 31].

Not only obesity linked microbiota characteristics, but also changes in microbiota composition during weight loss are under discussion. It has been suggested that the abundance of *Bacteroidetes* responds to the weight loss whereas the *Firmicutes* remain unaffected [32]. Recently published data show that especially *Firmicutes* promote caloric extraction from the diet in zebra-fish. Eating encourages the growth of *Firmicutes* and the fat storage in intestinal cells. *Firmicutes* decrease during hypocaloric diets and *Bacteroidetes* get the chance to proliferate [33]. Larsen et al shows a positive correlation between plasma glucose levels and *Bacteroidetes* to *Firmicutes* ratio [13]. As we approved the HbA1C blood levels of our type 2 diabetic patients, we had no possibility to approve this correlation. After the start of intervention with Victoza® all type 2 diabetics had well-adjusted HbA1C blood levels of 7-8 %, a value that according to American Diabetes Association protects against long-term consequences [34].

Higher Lactic Acid Bacteria (LAB) may contribute to chronic inflammation?

Our type 2 diabetes patients harbored higher proportions of LAB according to qPCR analysis. This was confirmed by sequencing for higher abundance of Enterococcus, Streptococcus and Lactobacillus. Furthermore, LAB increased in type 2 diabetics during the study period (figure 1).

LAB are very well known, but also subject of controversy [3], they represent a very heterogeneous group with well documented immune modulating qualities, which may potentially contribute to chronic inflammation in type 2 diabetes [13]. The relative abundance of *Bacilli* is increased in diabetics; especially the *Lactobacillus* group is enriched in type 2 diabetic mice models. Many bacterial isolates received from diabetic patients are able to produce toxines, indicating the necessity for virulence of these bacteria in blood stream infections [35]. On the other hand several strains of *Lactobacillus* are tested as probiotics, e.g. *L. paracasei ssp. paracasei* F19 and *L. gasseri* SBT2055 to decrease fat mass (visceral and subcutaneous).
Therefore, increases in LAB can be interpreted as immune modulating, even inflammatory process or an immune mechanisms underlying body-weight management.

Higher enterobacteria in type 2 diabetics - contributors to bacteremia?

Type 2 diabetics and to a lesser extent also obese volunteers showed a higher abundance of enterobacteria, especially *E. coli*. Enterobacteria are of special interest in type 2 diabetes as enterobacteria are a major cause of morbidity and mortality in type 2 diabetics [36]. Thomsen et al. observed a 3-fold increased risk to *E. coli* and other enterobacteria. Diabetic patients aged <45 years have a 15-fold higher risk for acute pyelonephritis and diabetics aged >65 had 3-6 times higher risk compared to non-diabetics. A diabetes prevalence of 20-30 % was previously reported among patients with enterobacterial bacteremia [37].

Caloric restriction increases abundance of *Akkermansia* and *Archaea*?

We report an enhanced abundance of *Akkermansia* (figure 2,3) and a higher appearance of *Archea* in the type 2 diabetes group. Only few lean controls harbored *Archaea*. On the basis of mucin-degradation *Akkermansia* do not compete with the other microbiota and are not dependent on nutrients deriving from host food consumption [38]. The observed higher abundance of *Akkermansia* in type 2 diabetics

in our study may reflect a better adaption to caloric restriction compared to the rest of intestinal microbiota as they do not depend on host diet. An increased abundance of *A. muciniphila* has also been observed in hamsters lacking food-derived enteral nutrients [39].

Archaea have been reported to be slightly more abundant in obesity and anorexic patients and were discussed to adapt towards optimal exploitation of hypocaloric diet [31]. Therefore, *Archaea* are indirect promoters of caloric intake by the colon and further fat accumulation [40]. Studies in humans show that removal of H2 by the synothropic relationship with bacteria improves fermentation efficiency [7, 41]. Moreover less energy has been measured in obese feces in mice relative to lean littermates [41]. The symbiotic relationship might maximize the microbiota's ability to generate energy from otherwise non-digestible food components, thereby they get enriched during caloric restriction to guarantee an adequate caloric intake of the host and as such complicate or even prevent weight reduction over time.

***Prevotella* reflect same compositional diet between the three groups**

Our study participants showed no differences in *Prevotella* abundance over time, as our type 2 diabetics were well adjusted to HbA1c blood levels. In contrast Larsen et al. reports significant higher ratios of *Bacteroides-Prevotella* group versus class *Clostrida* and *C. coccoides- E. rectale* group in type 2 diabetics. *Clostridium ssp., C. coccoides* decrease and *Bacteroides-Prevotella* group increase with body weight loss [13].

Prevotella are also of special interest because of their role in the formation of discussed gut enterotypes. First of all we made the distribution into the nowadays confirmed two enterotypes (*Bacteroides-Akkermansia* or *Prevotella*) with no observable difference. However, if we have a detailed view with the distribution of *Bacteroides* and *Akkermansia* in each enterotype, type 2 diabetics are more related to enterotype 3 and obese particpants and the lean control group to enterotype

1, which might reflect the dependence of an enterotype on caloric restriction although both are related to a Western diet.

On the other hand Oluf Pedersen indicates that obese and “metabolically unhealthy” people tend to have the *Bacteroides* enterotype, so the classification may get very important to ensure an individual therapy and treatment [42].

Conclusion

Gut microbiota composition and activity is a key element in controlling vital functions of the host and also the nutritional status. Type 2 diabetes and obesity are associated with changes in its abundance and diversity. Microbiota interact with functions underlying inflammation, energy extraction as well as the gut barrier. Our results indicate the necessity to concentrate on gut microbiota – host interaction to better understand the involvement of gut microbiota in the development of obesity and type 2 diabetes.

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Appendix

Tables

Table 1: Characterization of participant groups

Table 2: Primers and TaqMan®-probes targeting 16rRNA coding regions of bacteria and archaea

Table 3: Primers (SYBR® Green) targeting 16rRNA coding regions of bacteria, yeasts and butyrylcoenzyme A (CoA) CoA transferase genes

Table 4: Number of bands observed in PCR-DGGE fingerprinting in lean controls, obese patients and type 2 diabetics at all three time points

Figures

Figure 1 16S rDNA qPCR quantification with primer pair for lactic acid bacteria showing significant higher abundance in type 2 diabetics (D) than obese patients (OC) and lean controls (LC) (T1, samples taken before intervention; T2, samples taken after 1 month of intervention; T3, samples taken after 4 month of intervention)

Figure 2 Bar Charts of 454 sequencing results reflect qPCR analysis with increasing abundance of *Firmicutes* especially *Clostridiales* and *Bacilli* in type 2 diabetics (D) resulting in an increasing ratio of *Firmicutes*: *Bacteroidetes* between the time points (T1, samples taken before intervention; T3, samples taken after 4 month of intervention). *Verrumicrobia* and enterobacteria are also higher abundant in type 2

diabetics and enriched in obese patients (OC). Lean controls (LC) showed an according lower abundance of named bacterial groups and higher abundance of *Bacteroidetes*.

Figure 3 Heatmap showing abundances within the 454 sequencing dataset between the groups and time points (OC – obese patients, LC – lean control; D1 – type 2 diabetics time point one; D3-type 2 diabetics time point 3) The OTU heatmap displays raw OTU counts per sample, where the counts are colored based on the contribution of each OTU to the total OTU count present in that sample, blue indicates contributions with low percentage of OTUs to sample, whereas red contributes for a high percentage of OTUs.

Figure 4 PCA based on dominant bacteria of PC1 (*Firmicutes* and *Verrumicrobia)* and of PC2 (*Proteobacteria, Actinobacteria* and *Bacteroides)* illustrating grouping of type 2 diabetic samples (ellipse), as a result of a higher ratio of *Firmicutes: Bacteroidetes* in type 2 diabetics, lower abundance of *Verrumicrobia* and higher abundance of *Proteobacteria*

Figure 5 Jackknifing did not affect UPGMA clustering results as groups and patients are less different compared to differences between the three groups (LC, lean control group; OC, obese patients). Type 2 diabetics (D) are grouped between the two time points (T1, samples taken before intervention; T3, samples taken after 4 month of intervention).

Figure 1:

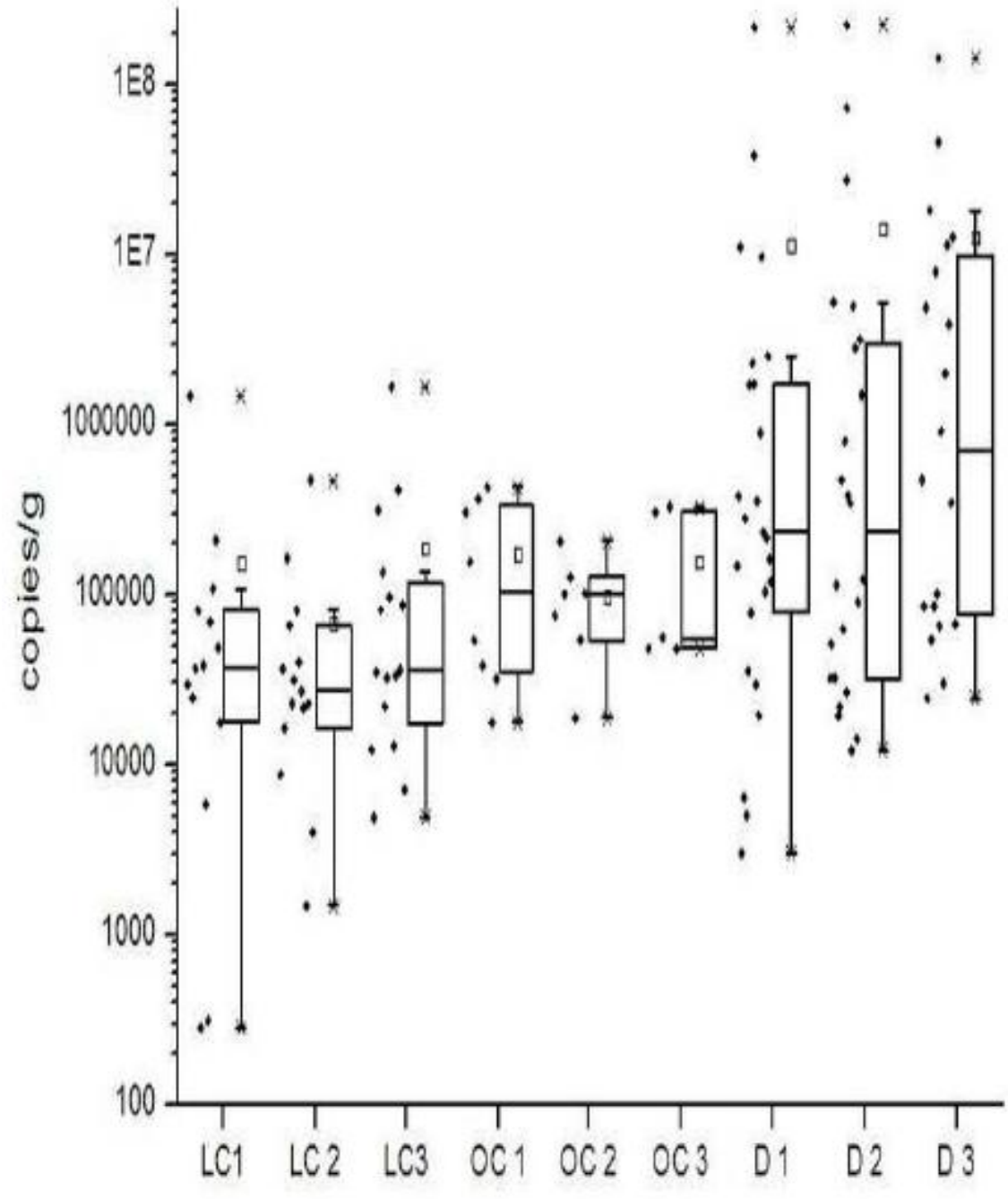
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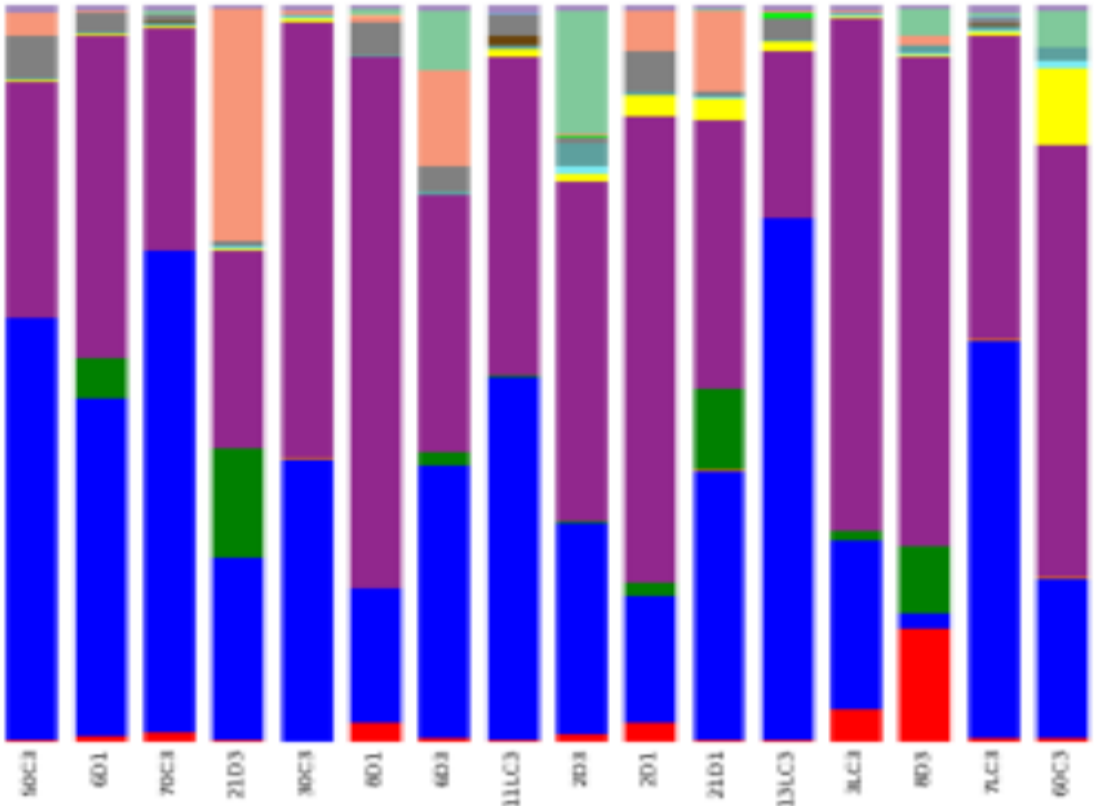
Figure 2:


Bacteria;Actinobacteria;Actinobacteria
Bacteria;Bacteroidetes;Bacteroidia
Bacteria;Bacteroidetes;Other
Bacteria;Firmicutes;Bacilli
Bacteria;Firmicutes;Clostridia
Bacteria;Firmicutes;Erysipelotrichi
Bacteria;Firmicutes;Other
Bacteria;Fusobacteria;Fusobacteria
Bacteria;Other;Other
Bacteria;Proteobacteria;Alphaproteobacteria
Bacteria;Proteobacteria;Betaproteobacteria
Bacteria;Proteobacteria;Deltaproteobacteria
Bacteria;Proteobacteria;Gammaproteobacteria
Bacteria;Proteobacteria;Other
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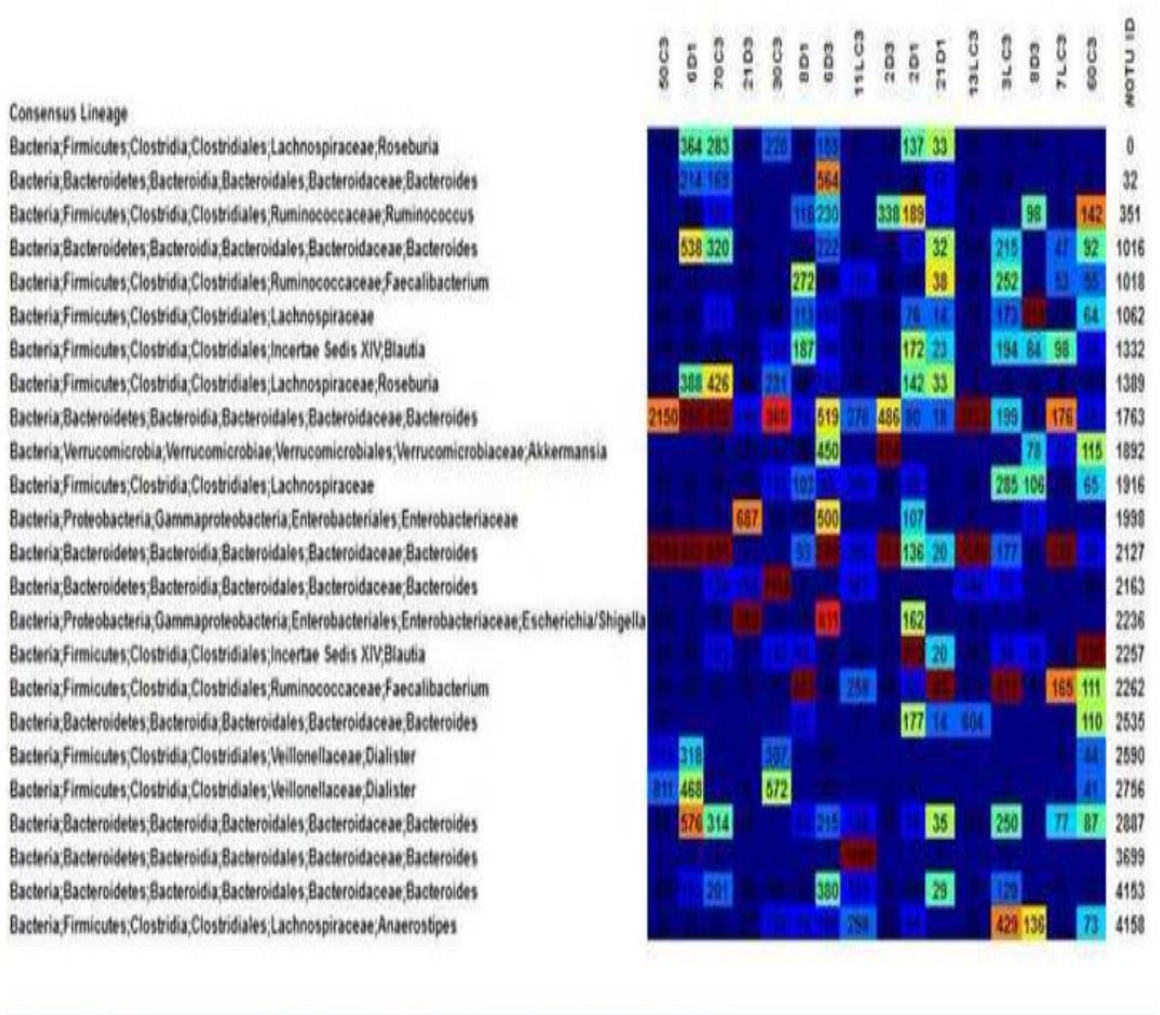
Unassignable;Other;Other
Unclassified;Other;Other

Figure 3: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8D3
7LC3
60C3
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Consensus Lineage
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Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides
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Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae;Dialister
Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae;Dialister
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Anaerostipes

Figure 4:

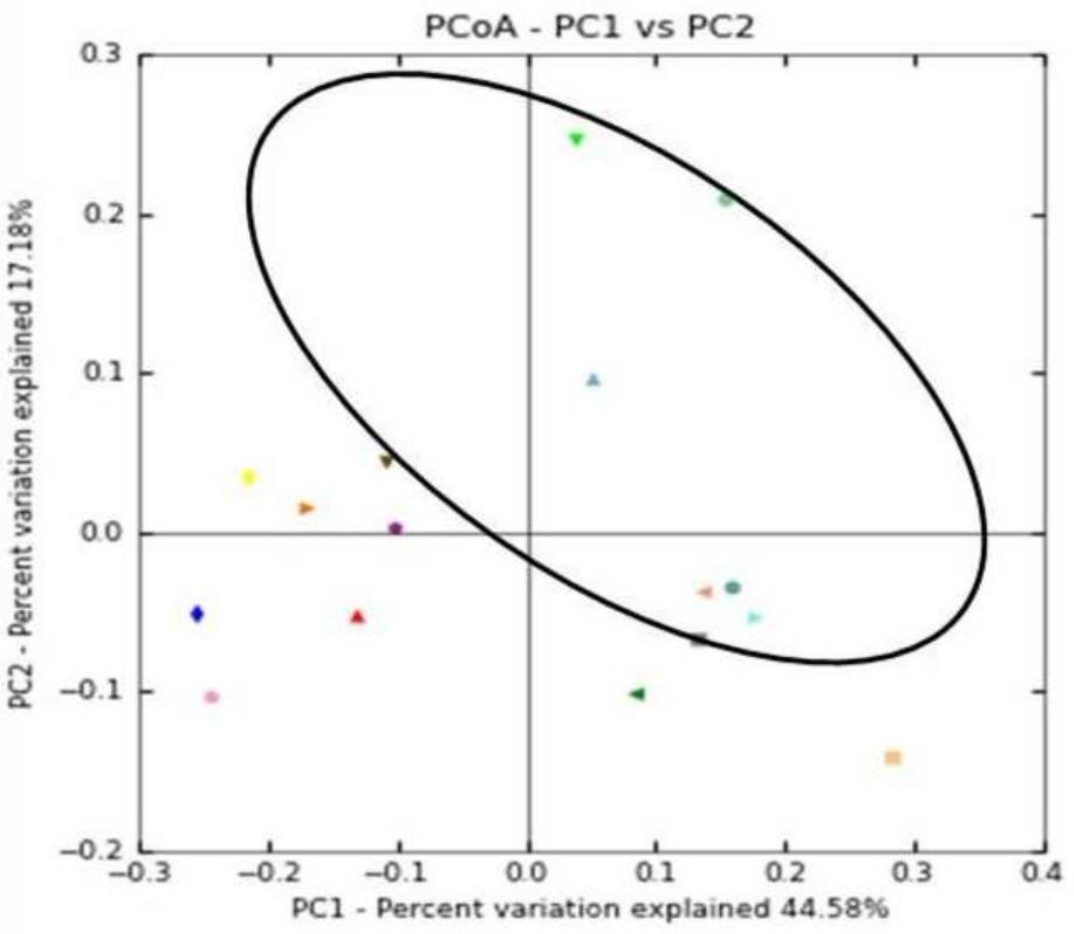
PCoA - PC1 vs PC2
PC2 - Percent variation explained 17.18%
PC1 - Percent variation explained 44.58%
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4

Figure 5:

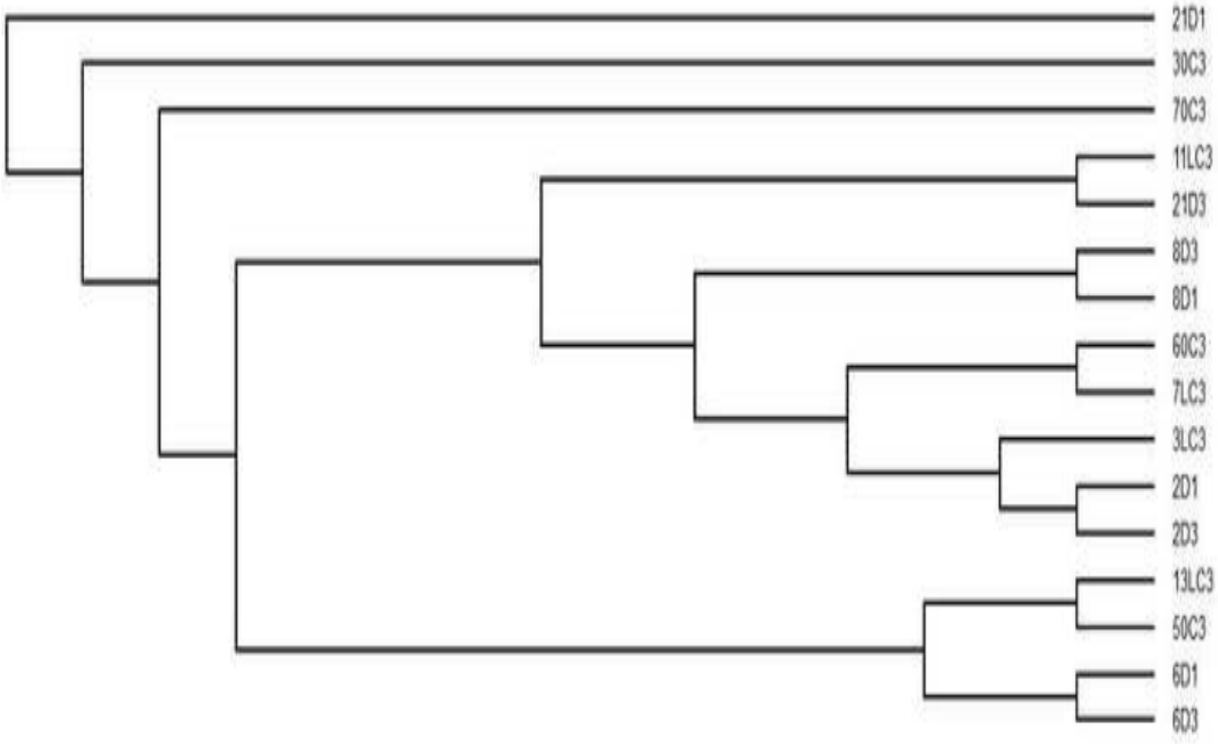
21D1
30C3
70C3
11LC3
21D3
8D3
8D1
60C3
7LC3
3LC3
2D1
2D3
13LC3
50C3
6D1
6D3

Table 1: Characterization of participant groups

Group	characterization	rate	BMI (± SD)
LC	Lean control group, no treatment	19	21,78 (± 2,35)
OC	Obese patients, intervention with nutritional counseling	14	33,71 (± 3,97)
D	Type 2 diabetic, treatment with nutritional counseling and GLP-1 Agonist	24	38,01 (± 5,81)

Table 2: Primers and TaqMan®-probes targeting 16rRNA coding regions of bacteria and archaea

Target organism	Primer/Probe	Sequence (5' - 3')	Size (bp)	Conc. [pmol/µL]	Reference
All *Bacteria*	Fwd primer	ACT CCT ACG GGA GGC AG	468	10	[1]
	Rev primer	GAC TAC CAG GGT ATC TAA TCC		10	
	Probe	(6-FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		2	
Clostridium* cluster IV *(Ruminococcaceae)	Fwd primer	GCA CAA GCA GTG GAG T	239	4	[2]
	Rev primer	CTT CCT CCG TTT TGT CAA		4	
	Probe	[3]-AGG GTT GCG CTC GTT-(BHQ-1)		2	
Cluster XIVa (Lachnospiraceae)	Fwd primer	GCA GTG GGG AAT ATT GCA	477	5	[4]
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A		5	
	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)		1,5	
Bacteroidetes	Fwd primer	GAG AGG AAG GTC CCC CAC	106	3	[5]
	Rev primer	CGC TAC TTG GCT GGT TCA G		3	
	Probe	(6-FAM)-CCA TTG ACC AAT ATT CCT CAC TGC		1	

		TGC CT-(BHQ-1)			
***Bifidobacterium* spp.**	Fwd primer	GCG TGC TTA ACA CAT GCA AGT C	125	3	[6]
	Rev primer	CAC CCG TTT CCA GGA GCT ATT		3	
	Probe	(6-FAM)-TCA CGC ATT ACT CAC CCG TTC GCC-(BHQ-1)		1.5	
Archaea	Fwd primer	ATT AGA TAC CCG GGT AGT CC		4	[7]
	Rev primer	GCC ATG CAC CWC CTC T	1044 – 1059	4	[1]
	Probe	(6-FAM)-AGG AAT TGG CGG GGG AGC AC(BHQ-1)	915– 934	4	

Table 3: Primers (SYBR® Green) targeting 16rRNA coding regions of bacteria and butyryl-coenzyme A (CoA) CoA transferase genes

Target organism	Primer	Sequence (5' - 3')	Size (bp)	Conc. [pmol/µL]	Reference
Lactic Acid Bacteria (LAB)	Fwd primer	AGC AGT SGG GAA TCT TCC A	352-700	4	[8]
	Rev primer	ATT YCA CCG CTA CAC ATG		4	
BCoAT gene	Fwd primer	GCI GAI CAT TTC ACI TGG AAY WSI TGG CAY ATG	~540	27	[9]
	Rev primer	CCT GCC TTT GCA ATR TCI ACR AAN GC		27	
Enterobacteria	Fwd primer	AGC ACC GGC TAA CTC CGT	492-509	3	[10]
	Rev primer	GAA GCC ACG CCT CAA GGG CAC AA	834 - 856	3	[11]
Prevotella	Fwd primer	CACCAAGGCGACGATCA	1458	2,5	[12]
	Rev primer	GGATAACGCCYGGACCT		2,5	
Akkermansia	Fwd primer	CAGCACGTGAAGGTGGG GAC	1505	2,5	[13]
	Rev primer	CCTTGCGGTTGGCTTCA GAT		2,5	

Table 4: Number of bands observed in PCR-DGGE fingerprinting

time point	LC	OC	D
T1	12,1 ± 3,5	10,5 ± 3,6	8,5 ± 4,4
T2	13,3 ± 4,9	9,0 ± 1,6	9,4 ± 3,4
T3	14,0 ± 4,0	10,3 ± 5,6	9,6 ± 3,6

(LC- lean controls; OC- obese participants and D type 2 diabetics; T – time point)

11.2. Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity

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Abstract

Gut microbiota and microbial metabolic activities of the lipid and glucose metabolism, satiety and chronic low-grade inflammation are known to be involved in the metabolic syndrome. Especially fermentation end products, SCFAs are believed to engage the epigenetic regulation of inflammatory reactions via FFARs and other SCFA-receptors.

We studied a potential interaction of microbiota with epigenetic regulation in obese and type 2 diabetes patients compared to a lean control group over a four month intervention period. Microbiota was analyzed for abundance, b*utyryl-CoA: acetate CoA-transferase* gene, and for diversity by qPCR, DGGE and 454 high-throughput sequencing. Epigenetic methylation of the promoter region of FFAR3 and Line-1 was analyzed using bisulfite conversion and pyrosequencing.

The diversity of the microbiota as well as *Faecalibacterium prausnitzii* were significantly lower in obese and type 2 diabetic patients compared to lean individuals. Results from *Clostridium cluster IV* and *Clostridium cluster XIVa* showed a decreasing trend in type 2 diabetics in comparison to the *butyryl-CoA: acetate CoA-transferase* gene and according melt curve analysis.

The analysis of the FFAR3 promoter region showed a significant lower methylation% in obese and type 2 diabetics with an increase in obese patients over intervention period. LINE-1, a marker of methylation, indicated no significant differences

between the three groups or the time points, although methylation of type 2 diabetics tended to increase over time. In addition we found a significant correlation between a higher BMI and lower methylation% of FFAR3.

Our results give evidence that a different composition of gut microbiota in obesity and type 2 diabetes affect the epigenetic regulation of inflammatory molecules. Interactions between microbiota and epigenetic regulation may involve SCFAs binding to FFARs but also NF-κB signaling from bacterial cell wall components or DNA.

Keywords

butyryl-CoA: acetate CoA-transferase gene – *Faecalibacterium prausnitzii* – FFAR3 – Line-1- GLP-1 Agonist

Abbreviations

BMI	body mass index
DGGE	denaturing gradient gel electrophoresis
FFAR	free fatty acid receptor
FFG	Austrian Research Promotion Agency
FFQ	food frequency questionnaire
FWF	Austrian Science Fund
GLP-1	glucagon-like peptide 1
Gpr	G-protein coupled receptor
HbA_{1c}	glycol hemoglobin
HDAC	histon deacetylase
IFN-γ	interferon-γ
IL	Interleukin
LCFA	long chain fatty acid
Line-1	long interspersed nuclear element 1

NF-κB	nuclear factor κB
PCA	principal component analysis
PGB	Porphobilinogen-Deaminase
PPAR-γ	peroxisome proliferator-activated receptor-γ
PYY	Peptide YY
SCFA	short chain fatty acid

Introduction

The gut microbiota plays a critical role in the establishment and maintenance of health. A wide range of inflammatory and metabolic diseases is associated with microbial imbalance [1,2]. Obesity has been associated with changes in abundance, diversity and metabolic function of the gut microbiota [2]. These compositional changes are indicated by a higher abundance of *Firmicutes* and a decreased abundance of *Bacteroidetes* [1,2,3]. However, controversial data [4] or no link of obesity to the ratio of *Firmicutes /Bacteroidetes* are also described [5]. A higher *Firmicutes* versus *Bacteroidetes* ratio is hypothesized to increase the fermentation end-products butyrate and acetate, as many *Firmicutes* are butyrate producers [6]. *Faecalibacterium prausnitzii* is well described for butyrate synthesis, anti-inflammatory activity, decreasing the rates of NF-κB (nuclear factor-κB) activation and IL-8 (interleukin) secretion, increasing IL-12, IL-10 and IFN-γ (interferon-γ), and also as an indicator of microbial diversity [7]. Evidence is accumulating that a higher diversity is related to health, whereas a lower diversity is observed in relation to various diseases [8]. The gut microbiota is also involved in obesity-associated metabolic disorders, type 2 diabetes, metabolic endotoxemia and low-grade inflammation [9]. Type 2 diabetes has often been associated with lower abundance of *Firmicutes*, while *Bacteroidetes* and *Proteobacteria* tended to be more abundant [1]. Controversial data showed lower abundance of *Bacteroidetes vulgatus* and *Bifidobacterium* spp. and a higher of *Clostridium leptum cluster* in di-

abetics compared to healthy volunteers [10]. In addition, the relative abundance of Bacilli is increased in diabetic mice models [1]. Our recently published data support these findings of a higher *Firmicutes /Bacteroidetes* ratio with the highest contribution coming from lactic acid bacteria. *Lactobacilli* together with *Enterobacteria* may contribute to the associated systemic low-grade inflammation of obesity and type 2 diabetes. Both are mentioned to induce inflammation via their immune modulating properties and to provoke the macrophagen-mediated adipose tissue inflammation [11].

A part of the microbiota, SCFA-producing bacteria are known to be involved in the fermentation of dietary fibers by converting sugars partially to short-chain fatty acids (SCFAs), including acetate, propionate and butyrate [12]. These SCFAs may help explain some mechanisms by which gut microbiota affect obesity and chronic diseases. They have a role in the regulation of energy metabolism, immunity and adipose tissue expansion [13]. On the other hand all three SCFAs are proposed to protect against diet-induced obesity, with butyrate and propionate being more efficient than acetate [14]. They are ligands of two different G-protein coupled receptors, GPR41 (FFAR3; free fatty acid receptor) and GPR43 (FFAR2). The affinity of SCFAs to FFAR3 and FFAR2 differs with the length of the fatty acids. FFAR3 prefers the ligands butyrate and propionate, whereas FFAR2 prefers acetate and propionate [14,15]. An additional receptor, GPR40, does not bind SCFAs but LCFAs (long chain fatty acid) and is located upstream very close to GPR41 gene. Halpern et al. mentions that GPR41 may has no promoter region and thus is transcribed by the promoter of GPR40 [16]. Biological functions of FFARs are the modulation of lipid metabolism, expression of the leptin gene and the control of food intake. Effects of SCFAs are also believed to include epigenetic control of gene expression [17]. Butyrate is well-known for its epigenetic activity as histone deacetylase inhibitor (HDACi). Modulation of histone acetylation by SCFAs may prevent obesity through anti-inflammatory and anti-carcinogenic effects. Activities of histone

deacetylases and methyl CpG binding proteins may influence DNA-methylation [18]. Folat is the best known methyl group donor, however other substances such as methionine, choline and butyrate are also able to influence the DNA methylation status. Changes in the CpG methylation of genes or promoter regions of inflammatory mediators or receptors regulate the expression. Highly methylated promoter regions are proposed to decrease transcriptional activity of the downstream gene [19]. In contrast, a global DNA methylation, often analyzed by the methylation of long interspersed nuclear element 1 (LINE-1) is discussed for DNA stability. LINE-1 elements are usually high methylated and their variability can be associated with cancer, age, ischemic heart disease and stroke as well as with plasma fasting glucose and plasma lipid levels [20].

We investigate differences of FFAR3, LINE-1 in human blood samples, *butyryl-CoA: acetate CoA-transferase* gene and compositional changes in *Firmicutes* of microbial DNA from feces of obese patients without established insulin resistance, type 2 diabetics under GLP-1 Agonist therapy (glucagon like peptide-1 Agonists) and a lean control group. The GLP-1 Agonist increases the glucose-dependent insulin secretion in beta cells and suppresses the glucagon secretion in the Pancreas. It also slows the gut passage in order to decrease the postprandial spike in blood glucose [21]. In addition, we investigated the response of gut microbiota and according epigenetic consequences to intervention and its potential role in controlling metabolic syndrome.

Materials and methods

Study participants and study design

The study “Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity” was approved by the Viennese Human Ethics committee. All study participants gave written consent for using blood and stool samples and data obtained from food frequency questionnaires (FFQ) for the analyses presented here. The FFQ asked participants to report the frequency of consumption and portion size over a defined period (last three months). Additional to dietary habits, the FFQ included questions about lifestyle (i.e. smoking, alcohol consumption, physical activity), medical relevant influences (i.e. vitamin supplements and additives), BMI (body mass index) and age at enrollment. To ensure comparable data, patients were interviewed for their history of gastrointestinal diseases, use of antibiotics, probiotics or prebiotics.

We studied fourteen obese (OC) with no established insulin resistance (aged 38 +/- 14 years; BMI 33,71 ± 3,97 kg/m^2) and twenty-four insulin-dependent type 2 diabetes (D) patients (aged 56 +/- 9 years; BMI 38,01 ± 5,81 kg/m^2) over a four month intervention period and compared to a lean control (LC) group (n = 19; aged 30 +/- 8 years; BMI 21,78 ± 2,35 kg/m^2). Intervention comprises GLP-1 Agonist for type 2 diabetics and nutritional counseling for both intervention groups.

Blood and fecal samples were collected at three occasions; before treatment, during and after treatment, with a distance of 1 month between the first two time points and 3 months between the second and third time point. HbA_{1C} (glycol hemoglobin) blood level was determined at laboratory Health Center South in Vienna. The levels were between 7-8 mmol/mol hemoglobin in our type 2 diabetes group, a value that is according to American Diabetes Association protective against long-term consequences.

Fecal sample collection, processing, and analysis

Stool samples were stored at -70 C after collection. Bacterial DNA was extracted from fecal samples using the QIAamp® DNA Stool mini kit (Qiagen, Germany) according to the manufacturer's protocol. Additional, samples were treated in FastPrep™ Lysing Matrix E tubes (MP Biomedicals, USA) twice for 45 sec in a bead-beater (Mini-Beadbeater 8 Bio-Spec Products, USA) with an intervening minute on ice. DNA concentration and quality was determined with a Pico100 (Picodrop, UK) and agarose gel-electrophoresis.

The total bacterial diversity was measured by DGGE (denaturing gradient gel electrophoresis) using the primer set 341f-GC 5'-CCT ACG GGA GGC AGC AG-3' [22] and 518r 5'-ATT ACC GCG GCT GCT GG-3' [23] according to Remely et al. (2013) [11].

For further analysis in qPCR, we created clone libraries from dominant members of *Faecalibacterium prausnitzii* in two stool samples of healthy volunteers. Cloning was done following Remely et al. 2012 [11]. We quantified bacterial abundance with TaqMan qPCR and SYBR Green qPCR in a Rotorgene 3000 (Corbett Life Science, Australia) using 16S rDNA group specific primers (Table 1). *Butyryl-CoA:acetate CoA-transferase* gene was amplified and thermal denatured with SYBR Green qPCR analysis. The amplicons were divided into three areas, as described by Louis & Flint (2009). These peaks are assigned to represent bacteria related to *Eubacterium hallii* and *Anaerostipes coli* (82.5–85.0 1C), *Roseburia/E. rectale spp.* (85.5–89.0 1C) and *F. prausnitzii* (89.5–92.5 1C).

The specificity of primer and probes was checked with the ProbeMatch function of the ribosomal database project 10 (http://rdp.cme.msu.edu/). The PCR reactions mixture and serial DNA dilution of typically strains were prepared according to Pirker et al. 2012 [24].

Sixteen samples were analyzed with Roche GS FLX + Titanium next generation sequencing (LGC sequencing GmbH, Germany). For alignment and further anal-

yses we used the pyro pipeline of the ribosomal database project 10 (http://rdp.cme.mus.edu/) and qiime. We then performed significance tests and principal coordinate analysis in qiime.

Blood sample collection, processing, and analysis

Blood samples were collected in PAXgene Blood DNA Tubes (Qiagen, Germany) and stored at -20°C. For extraction of DNA, they were treated with the PAXgene Blood DNA Kit (Qiagen, Germany). For epigenetic analysis the DNA was converted with the EpiTect Bisulfite Kit (Qiagen, Germany) according to manufacturer's protocol. The DNA was measured and approved for purity with a Pico100 (Picodrop, UK) and agarose gel-electrophoresis. Suitable primers for the selected 5 CpGs in the promoter regions of FFAR3 (Table 1-2) were designed using the PyroMark Assay Design Software 2.0 (Qiagen, Germany). The PCR was carried out in a total reaction volume of 25 µL, it contains 12,5 µL Pyromark 2X PCR master mix, 5 pmol (FFAR3) or 1 pmol (LINE-1) of each primer, 2,5 µL Coralload Concentrate 10X (Qiagen,, Germany), and 5 ng (FFAR3) or 10 ng (LINE-1) bisulfite converted DNA. PCR product quality was investigated with agarose gel-electrophoresis. Analysis of CpG methylation was performed with a PyroMAark Q24 MDx (Qiagen, Germany).

Statistical analysis

The normality of distribution was approved with the Kolmogorov-Smirnov test. The comparison of gut microbiota was performed using the non-parametric Mann-Whitney U test and for three unpaired groups the non-parametric Kruskal Wallis ANOVA. Differences in the percent of methylation of LINE-1 and FFAR3 were determined using the student's two tailed T-test, one-way analysis of variance (ANOVA) and with the Tukey post hoc correction test. For correlation analysis we used the two-tailed Pearson test. P values < 0.05 were determined as statistically significant. All data shown are mean ± sd.

Results

Analysis of the FFQ revealed differences in life style patterns and in the consumption of sugary products. At all three time points the total sweet consumption was the lowest in type 2 diabetics. In contrast obese patients showed the highest intake, 50 % of participants consumed sweets one to three times per week. No differences were observed in the consumption of grain, vegetables, fruits, meat, dietary products and fish. In life style patterns type 2 diabetics showed the lowest physical activity (64.0 % T1, 52.0 % T3 never do sports), followed by obese participants (37.5 % T1, 12.5% T3 never do sports), whereas 44.0 % of lean controls exercised 1-3 times per week. 19 out of 24 type 2 diabetes patients showed an average weight reduction of 3.1 ± 2.1 kg, obese patients lost about 4.9 ± 3.7 kg (Table 3).

DGGE fingerprinting analysis of total bacteria revealed significant differences between type 2 diabetics and lean controls at time point 2 ($p = 0.03$) and time point 3 ($p = 0.01$). Lean controls showed the highest diversity, followed by obese participants and by type 2 diabetics showing the lowest diversity. Although the first two principal components (PCs) explained 15.6 % and 12.0 % of variance (data not shown), principal component analysis (PCA) showed grouping of band patterns according to the three groups of study participants.

Lean controls had a higher Shannon's diversity index (6.48 ± 0.47) of 454 sequencing results and a higher number of sequences (683.3 ± 59.6) compared to obese controls (4.51 ± 2.60; 571.5 ± 48.2) and type 2 diabetics (5.19 ± 2.62; 645.5 ± 22.6). A clustering of the participants in the PCA revealed grouping of type 2 diabetes subjects. The first three calculated PCs of 454 sequencing results explained 44.6 %, 17.2 % and 8.3 % of the variance in the data. *Firmicutes* and *Verrumicrobia* contributed most to the separation along PC1 and *Proteobacteria, Actinobactera* and *Bacteroides* contributed to the clustering along PC2.

We focused on groups of *Firmicutes*, known for their high number of SCFA-producing bacteria. *Clostridium Cluster IV* and *Clostridium Cluster XIVa* abundances were not significantly different between the groups or intervention period but tended to decrease with intervention in type 2 diabetics. High throughput sequencing confirmed these findings as type 2 diabetics were enriched in *Firmicutes* especially *Clostridiales* and *Bacilli*, in particular *Lactobacillales* as described before. Quantitative PCR analyses of *Faecalibacterium prausnitzii* go in line with high throughput sequencing analysis. Type 2 diabetics showed a significantly lower abundance at all three time points with an improvement at the third time point. The lean control group showed the highest abundance. These findings were significant at all three time points between lean control group, type 2 diabetics and obese patients (T1 $p < 0{,}001$; T2 $p < 0{,}001$; T3 $p < 0{,}001$). In addition, obese participants showed a higher abundance compared to type 2 diabetics. No significant changes within a group were observed over the study period (Figure 1).

The b*utyryl-CoA: acetate CoA-transferase* gene was used as a marker for butyrate production as the direct analysis of SCFAs in feces is difficult by rapid binding and degradation of SCFAs in the gut. *Butyryl-CoA: acetate CoA-transferase* gene tended to increase in type 2 diabetics over the intervention period from an already lower level compared to the lean control group. Dominant butyrate producers differ in their DNA G+C content which was shown by the analysis of the melt curves obtained from PCR amplicons of the *butyryl-CoA:acetate CoA-transferase* gene. Detailed analysis allowed to differentiate between *Faecalibacterium prausnitzii, Eubacterium hallii* and *Roseburia.* We observed no significant differences in between our study population, although *Faecalibacterium prausnitzii* showed a trend to increase in type 2 diabetics.

Analysis of 5 CpGs in the promoter region of FFAR3 gene showed a significant higher methylation in the lean control group compared to type 2 diabetics at all three time points (T1 $p = 0.003$; T2 $p = 0.011$; T3 $p = 0.0001$) and to obese partici-

pants at the first time point (p= 0.031; Table 4, Figure 3). In type 2 diabetics, we observed no significant changes in the methylation during the intervention period (T1: 31.46 ± 5.74 %; T2: 32.53 ±7.17 %; T3: 31.25 ± 6.85 %). In comparison, obese patients showed an increase of methylation with nutritional counseling (T1: 30.69 ± 6.46 %; T2: 32.34 ± 7.13 %; T3: 34.51 ± 2.93 %), although they do not reach the methylation status of the lean control group (39.23 ± 0.9 %). The mean methylation of LINE-1 indicates no significant differences between the groups or the time points but obese individuals had the lowest methylation. We found a significant negative correlation between the BMI and methylation of FFAR3 (R= -0,474; p=0,001; Figure 4), whereas LINE-1 showed a tendency of a positive correlation. We observed no correlation between methylation of FFAR3 or Line-1 and age.

Discussion

We analyzed subgroups of the phyla *Firmicutes*, with a focus on the diversity, *Faecalibacterium prausnitzii* as a marker of inflammation, and the *butyryl-CoA: acetate CoA-transferase gene* as a proxy for SCFA-producers. Epigenetic methylation analysis included the CpGs in the promoter region of FFAR3 and LINE-1 as an indicator of the overall methylation. The results are discussed for the influence of SCFAs on epigenetic control of gene expression, especially of FFARs.

DGGE fingerprinting showed a lower diversity in type 2 diabetics compared to obese patients and lean controls. These findings were significant between lean controls and type 2 diabetics. However, we did not observe significant differences in Shannon's diversity index of our selected subgroup in 454 sequencing results. Diversity as an indicator of health and disease is still under discussion as other research groups do not observe significant differences between type 2 diabetics and non-diabetics [10] or even higher diversity and richness in diabetics with higher BMI (BMI > 31) compared to lean diabetics. In agreement with our observed PCs, PC1 *Firmicutes* and *Verrumicrobia*, PC2 *Proteobacteria*, *Actinobacteria* and *Bac-*

teroidetes, Larsen et al., 2010 also shows a separation between the groups attributed to *Bacteroides*, *Firmicutes* and *Verrumicrobia* (45 %) and in the second direction the combination of *Proteobacteria* and *Actinobacteria* (28 %) [1].

The analysis of one of the highest butyrate producing bacteria showed a lower abundance of *Faecalibacterium prausnitzii* in type 2 diabetics followed by obese patients (figure 1). However, our analyses of butyrate producing bacteria indicate no significant differences between type 2 diabetics and obese patients or the lean control group. In addition, analysis of *Clostridium cluster IV* and *Clostridium cluster XIVa* are in line with analysis of *butyryl-CoA: acetate CoA-transferase* gene. Thus we might have changes of species or even genus without a detectable outcome in the family itself. *Faecalibacterium prausnitzii* is identified as a dominant and conserved species in healthy human gut microbiota and is described to play a role during low-grade inflammation pathology [25]. A relationship between *Faecalibacterium prausnitzii* and inflammatory markers was observed in obese and diabetic patients and remained after adjustment for BMI. The proportions of *Faecalibacterium prausnitzii* were lower in type 2 diabetic subjects indicating a higher incidence of low-grade inflammation [7].

Melting curve analysis of *butyryl-CoA: acetate CoA transferase* gene and qPCR analysis of *Faecalibacterium prausnitzii* showed no clear correlation. One cause might be the described two pathways of butyrate production. The most important pathway is the *butyryl-CoA: acetate CoA-transferase*, using acetate as co-substrate. A minority of bacteria is able to use the *butyrate kinase* or *phosphotransbutyrylase* as an alternatively final step [26]. The used approach only detects bacteria which use the *butyryl-CoA: acetate CoA-transferase* pathway. Additional, butyrate producers do not form a homogenous phylogenetic group as butyrate producers and non-butyrate producers are in the same phylogenetic cluster

and although they are carrier of the gen there is no evidence for expression. Turnbaugh et al. (2006) estimated the role of gut microbiota profiles in the production of SCFAs at two levels: the capacity to produce SCFAs and the type of SCFAs produced [3]. Dependent on the initial SCFAs concentration status, there is evidence that inter-individual differences in polysaccharide fermentation are able to affect intervention with complex carbohydrates. Significant correlations are observed with gender, dietary factors, especially resistant starch and BMI. Dietary components and prebiotics affect also the growth of butyrate producers, for example the consumption of inulin stimulates the growth of *F. prausnitzii* and *E. rectale* [27]. Hippe et al. (2011) showed the important influence of diet or age on the butyrate production in the GI tract. The microbiota of the elderly is characterized by decreased butyrate production capacity, reflecting increased risk of degenerative diseases [26].

The absorption of SCFAs together with their conversion to fat in the liver and the regulation of host genes that promote deposition of fat in lipocytes, cause an increase in adipose tissue [28]. Butyrate in particular has an important impact on promoting body energy expenditure via a modulation of mitochondria function. The results are consistent with the role of SCFAs as an additional energy source promoting adiposity. Research of human SCFA content in feces indicated a higher proportion of SCFAs in overweight and obese participants compared to lean controls although the ratio of *Firmicutes /Bacteroidetes* is lower in overweight and obese [4]. A higher concentration of butyrate and acetate was measured in the caeca of obese mice, while propionate was not significantly different between the lean or obese group [3]. A higher ratio of *Firmicutes /Bacteroidetes* increases the energy harvest and the content of SCFAs and as such the lipogenesis and accumulation in adipocytes [29]. In contradiction, high-fat diets supplemented with butyrate prevent and also reverse insulin resistance in obese mice [30,31].

However, insufficient data currently prevent an estimation of optimal levels of fecal SCFAs for the promotion of colonic health. These observations support the hy-

pothesized engagement of SCFAs on host inflammatory signaling processes via epigenetic control of gene expression [13]. However, the precise role of SCFAs in signaling pathways is still unclear [30].

We observed a higher methylation in the promoter of the FFAR3 gene in the lean control group. Both intervention groups had a lower methylation with the lowest in type 2 diabetics (table 4, figure 3). These differences of methylation between the groups result in a negative correlation between methylation and BMI (figure 4). Type 2 diabetics showed no significant differences over the intervention period, whereas obese patients showed an increase of methylation after nutritional counseling. Thus a lower expression of FFAR3 in the lean control group can be expected according to Canani et al. (2011). In mice an increase of leptin production caused by an over expression of FFAR3 after oral doses of propionate was observed [32]. A knockdown of FFAR3 by siRNA inhibits the ability of propionate to induce leptin expression mainly in adipocytes [33]. Leptin itself takes influence on the hunger-satiety-cycle by suppression of the appetite and contribution to the fat metabolism. Although obese patients harbor many fat cells with according higher leptin levels, leptin is no longer able to contribute to satiety. It was discussed that high leptin levels over time make the satiety cells insensitive to leptin, whereas a supplementation with SCFAs induces sensitivity or the production of leptin. Only few obese patients are effected from leptin deficiency, so therapies using recombinant leptin have not been successful [34] and might affect therapies with SCFAs. As we observed a negative correlation of FFAR3 methylation and BMI, a lower methylation of the promoter region in obese and type 2 diabetics may indicate the proposed interaction of FFAR3 expression and higher leptin levels. On the other hand FFARs are discussed to stimulate the secretion of satiety hormone PYY (Peptide YY), which inhibits gut motility, slows intestinal transit time and enhances nutrient absorption.

These results are underlined by a study between Gpr41-deficient mice and conventional raised mice. A deficiency of the receptor is associated with a lower expression of PYY and as a consequence with a higher energy harvest from diet [35]. However, current researches indicate a FFAR3 independent influence of butyrate and propionate on body weight and food intake. In addition FFAR3 is mentioned to play a minor role in butyrate stimulation of GLP-1 secretion. SCFAs increase the reduced GLP-1 secretion of obese and type 2 diabetics and thus promote insulin secretion [14].

Therefore, a different microbial composition may contribute to reduced GLP-1 release. The main production of GLP-1 is located in the mucosal L cells, in the ileum and colon but also in pancreatic α-cells and in neurons [2]. Prebiotic treatment induced GLP-1 secretion in ob/ob mice, whereas a deletion of GLP-1 prevented the beneficial effects of prebiotics on weight gain, glucose metabolism and inflammatory pathway activation [35]. However, there is no evidence, that an intervention with GLP-1 Agonists for adjustment of blood glucose levels influences epigenetic methylation.

Changes in global methylation can affect gene expression, genomic stability, and chromosomal structure. In the case of LINE-1 methylation of our type 2 diabetics and lean controls showed the same methylation status, whereas obese patients had the lowest methylation. Baccarelli et al. (2010) associated blood DNA hypomethylation with higher risk of cardiovascular disease caused by older age, smoking, exposure to air pollution, folate deficiency, hyperhomocysteinanemia and end-stage renal disease [36]. Turcot et al. (2012) revealed lower LINE-1 methylation levels in visceral adipose tissue especially in obese subjects without established insulin resistance with an association to higher plasma glucose level, lower HDL-cholesterol level and greater risk to be affected by metabolic syndrome [20]. The incidence, that our obese study population has a lower global methylation than our

type 2 diabetics might be related to the well-adjusted HbA_{1c} value as a result of GLP-1 Agonist, whereas a higher blood glucose level in obese participants could not be excluded.

Conclusion

Our results suggest that a different composition of gut microbiota in obesity and type 2 diabetes effect the epigenetic regulation of inflammatory molecules, possibly also underlying a progression of the metabolic disease. Interactions between microbiota and epigenetic regulation may influence satiety and hunger cycle. Especially butyrate may help to develop strategies for regenerative medicine by promoting epigenetic remodeling and the expression of associated genes. Our results indicate the necessity to concentrate on gene-specific methylation to better understand the involvement of epigenetic regulation in the development of obesity and type 2 diabetes.

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Tables

Table 1: Primers and TaqMan®-probes targeting 16S rDNA coding regions of bacteria, BCoAT-gene and for CpG-Methylation analysis

Target	**Primer/Probe**	**Sequence (5' - 3')**	**Conc. [pmol/µL]**	**Reference**
Clostridium cluster IV (Ruminococcaceae)	Fwd primer	GCA CAA GCA GTG GAG T	4	[37]
	Rev primer	CTT CCT CCG TTT TGT CAA	4	
	Probe	(6-FAM)-AGG GTT GCG CTC GTT-(BHQ-1)	2	
Clostridium cluster XIVa (Lachnospiraceae)	Fwd primer	GCA GTG GGG AAT ATT GCA	5	[38]
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A	5	
	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)	1.5	
Lactic Acid Bacteria (LAB)	Fwd primer	AGC AGT SGG GAA TCT TCC A	4	[39]
	Rev primer	ATT YCA CCG CTA CAC ATG	4	
***BCoAT* gene**	Fwd primer	GCI GAI CAT TTC ACI TGG AAY WSI TGG CAY ATG	27	[12]

	Rev primer	CCT GCC TTT GCA ATR TCI ACR AAN GC	27	
Faecalibacterium prausnitzii	Fwd primer	GGA GGA AGA AGG TCT TCG G	0.25	[40]
	Rev primer	AAT TCC GCC TAC CTC TGC ACT	0.25	
FFAR3	Fwd primer	GTG ATA GGG AAA ATA GTA GTT GGT A	0.5	
	Rev primer	*Biotin*-CCC TAC AAA ACA AAA CTC ATC ATA AT		
	Seq primer	GGG AAA ATA GTA GTT GGT AT	0.4	
LINE-1	Fwd primer	TTT TGA GTT AGG TGT GGG ATA TA	1	[41]
	Rev primer	*Biotin*-AAA ATC AAA AAA TTC CCT TTC		
	Seq primer	AGT TAG GTG TGG GAT ATA GT	0.4	

Table 2: Sequence to analyze: FFAR3 (geneID (NCBI) 2865) and LINE-1

Assay	**Sequence 5´-3´**	**Number of CpGs**	**Size**
LINE1	TTC GTG GTG CGT CGT TT	3	17
FFAR3	CCG CTG CCT GCT CAC GAC CAC ACG CCA GGC TTC CGT CAA ACC ACT CAA CAT GTA TTA G	5	58

Table 3: Characterization of study participants

Group	**Age ± SD (years)**	**Time point**	**BMI ± SD (kg/m^2)**
D	58,36 ± 9,35	1	38,44 ± 5,14
		3	37,63 ± 5,58
OC	39,63 ± 15,18	1	33,71 ± 4,24
		3	32,66 ± 3,75
LC	25,67 ± 3,09	1	21,19 ± 1,93
		3	20,63 ± 1,87

Table 4: Methylation of 5 CpGs in the promoter region of FFAR3. At the first time-point (T1) lean controls (LC) showed highest methylation over all 5 CpGs, the lowest methylation has been observed in type 2 diabetics (D) and obese patients

GroupT 1	**CpG 1**	**CpG 2**	**CpG 3**	**CpG 4**	**CpG 5**	**Σ: CpGs**
D	37,29 ± 12,10	20,47 ± 5,22	24,48 ± 5,71	41,27 ± 7,85	33,82 ± 7,95	31,46 ± 5,74
OC	37,80 ± 14,32	18,78 ± 5,09	24,89 ± 3,78	37,13 ± 9,16	34,84 ± 6,31	30,69 ± 6.46
LC	42,86 ± 13,25	23,66 ± 3,95	27,21 ± 3,61	51,70 ± 6,46	46,13 ± 11,13	38,19 ± 6,19

Figures

Figure 1: 16S rDNA qPCR quantification with primer pair for *Faecalibacterium prausnitzii.* A significant ($p < 0.01$) lower abundance of *Faecalibacterium prausnitzii* was shown in type 2 diabetics (D) compared to obese patients (OC) and lean controls (LC) at all three time points

T1, samples taken before intervention; T2, samples taken after 1 month of intervention; T3, samples taken after 4 month of intervention

Figure 2: Bar Charts of 454 sequencing results reflect qPCR analysis. The abundance of *Firmicutes* especially *Clostridiales* and *Bacilli* in type 2 diabetics are increasing (D) between the time points. *Faecalibacterium prausnitzii* is significant higher abundant in lean controls (LC). In addition obese patients (OC) have a higher abundance compared to type 2 diabetics.

T1, samples taken before intervention; T3, samples taken after 4 month of intervention

Bacteroidales

Clostridiales, Faeclibacterium

Clostridiales, Blautia

Erysipelotrichales

Bacilli, Streptococcus

10

Figure3: Mean methylation of FFAR3 promoter region between the three groups at the first time point. Lean controls (LC) showed highest methylation compared to both intervention groups. These differences were significant between lean controls and type 2 diabetics (D, $p = 0.003$) and between obese participants (OC) and lean ($p= 0.031$).

Figure 4: Correlation between BMI and FFAR3methylation%. Significant correlation between a higher BMI of all study participants and lower FFAR3 methylation% at time point 1 (T1), before intervention (R = -0,474; p = 0,001).

Figure 1:

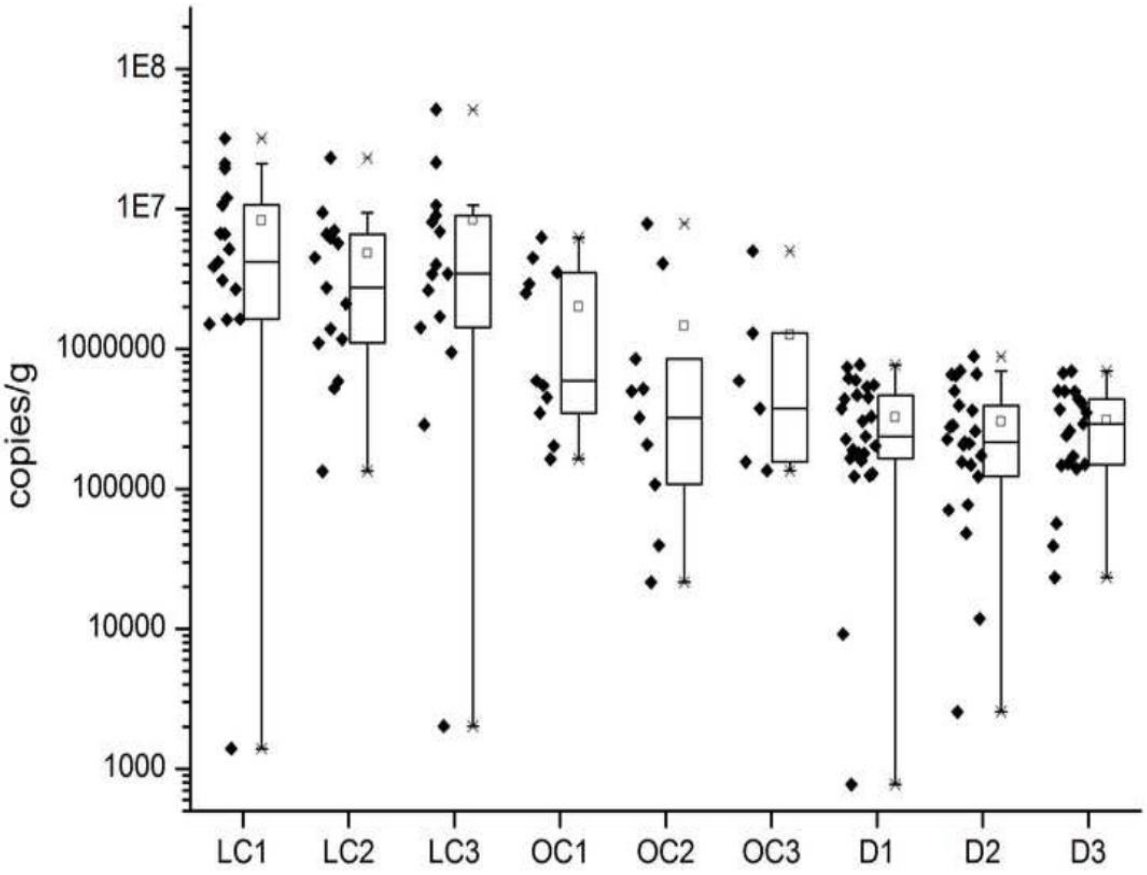


Figure 2:

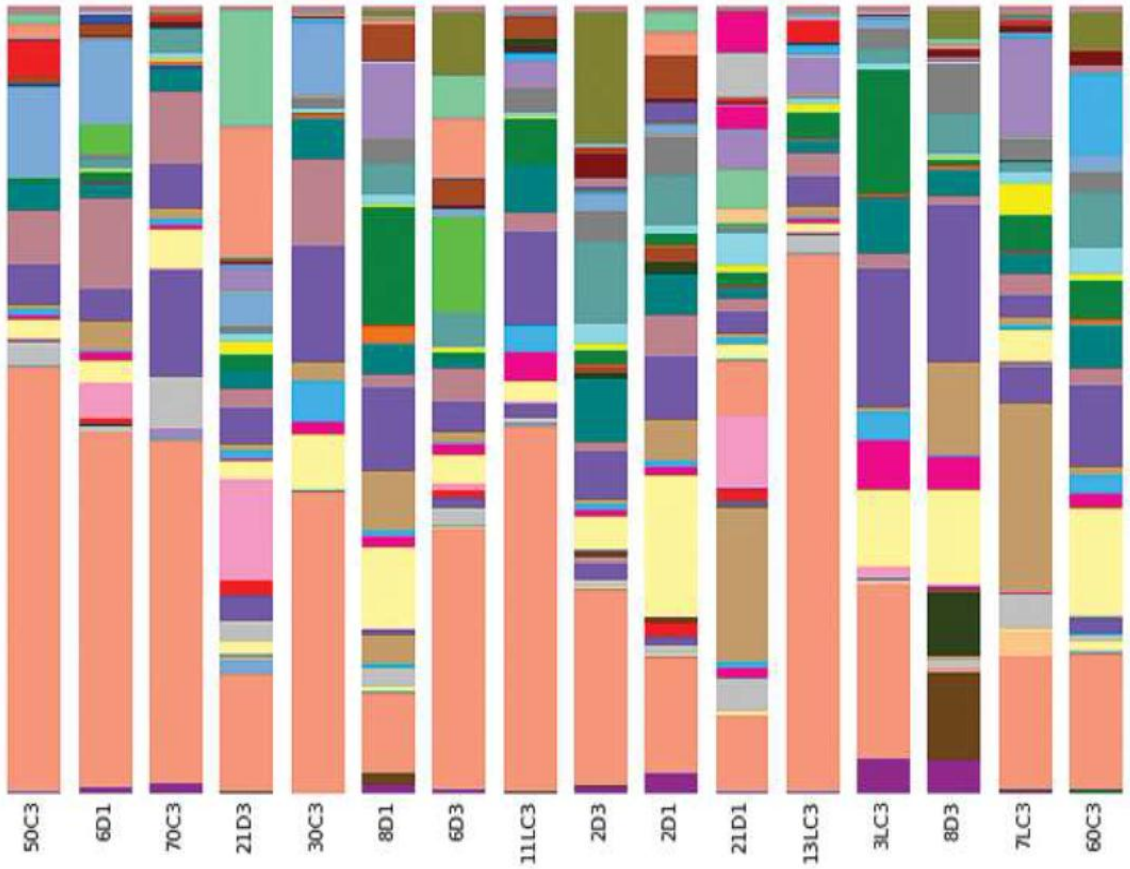

50C3
6D1
70C3
21D3
30C3
8D1
6D3
11LC3
2D3
2D1
21D1
13LC3
3LC3
8D3
7LC3
60C3

Figure 3:

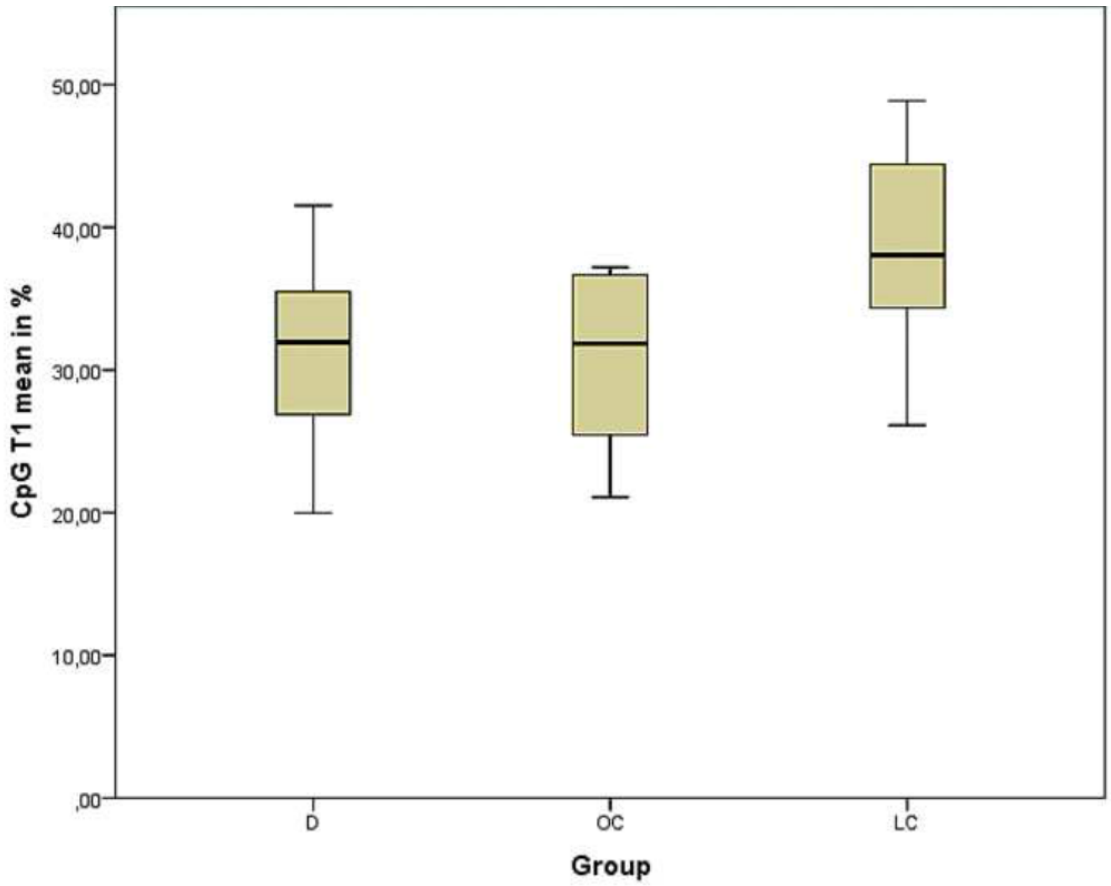
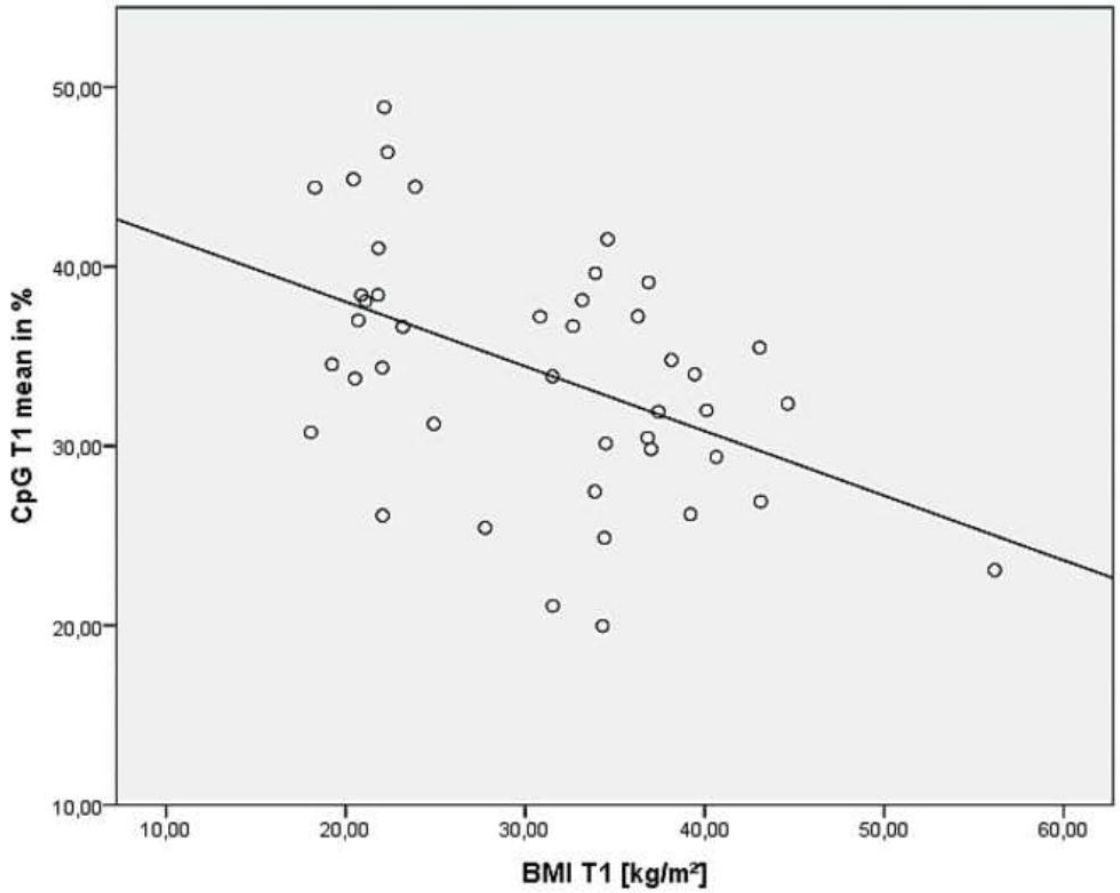


Figure 4:


CpG T1 mean in %
50,00
40,00
30,00
20,00
10,00
10,00
20,00
30,00
40,00
50,00
60,00
BMI T1 [kg/m²]

12. Abstract und Poster

12.1. Microbiota and epigenetic regulation of inflammatory mediators in metabolic syndrome and caloric restriction

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Diversity, shifts in subgroups and functions of microbial groups effect mechanisms involved in inflammation, ageing, metabolic diseases or central nervous diseases. Mediators from bacteria (SCFAs) and the interaction of bacteria or bacterial cell wall components (LPS), stimulate immune reactions and signaling pathways, for example toll like receptors (TLRs). The metabolic syndrome is a condition in which host genetic factors, microbiota composition and microbiota-directed regulation of gene expressions contribute to the pathogenesis in a combined way.

We analyzed changes in the GI-microbiota of type 2 diabetic (n = 25) volunteers under caloric restriction in comparison to lean (n = 18) and obese (n = 8) healthy controls. The abundance of bacteria and bacterial subgroups we measured in fecal samples with quantitative PCR (qPCR) of 16S rDNA coding regions. The methylation of TLR4 and TNF-α in blood mononuclear cells was sequenced with promoter specific primers for CpG-Methylation in a pyrosequencer.

The abundance of Lactobacilli and Clostridium cluster XIVa differed significantly in type 2 diabetics compared to lean controls before and after weight loss. In type 2 diabetics with weight loss the ratio of Firmicutes to Bacteroidetes increased throughout the study period. Opposite a decrease was shown in obese individuals with unintended weight loss. Archaea were significantly more abundant in individuals suffering from type 2 diabetes.

In addition to changes in the microbiota composition, we also report that epigenetic mechanisms regulate methylation of TLR4 and TNF-α. All analysed CPGs for TLR4 show decreased methylation in obese controls compared to lean controls and T2D. Methylation of CPGs of TNFa show only slightly decreased methylation in obese patients.

There is evidence that changes in the microbiota subgroups affect sets of TLRs which regulate inflammatory responses underlying obesity as well as type 2 diabetes.

4 th ÖGMBT Annual Meeting – Austrian Association of Molecular Life Science and Biotechnoolgy, Graz, September 17 – 19, 2012

3rd International Workshop Gut microbiota in Health and Disease, Sepember 13th -15th, Maastricht, Netherlands

Epigenetics Europe, September 4th -05th 2012, Frankfurt/Main, Germany

12.2. Relation of *Faecalibacterium prausnitzii* phylotypes and epigenetic regulation in obese, diabetic patients and controls

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Background: *Faecalibacterium prausnitzii* is one of the most abundant commensal bacteria and one of the main butyrate producers in the healthy human gut. Information on their genetic diversity and role in the inflammatory genesis is limited. Epigenetic regulation via FFAR or NFκB signaling are discussed.

Objectives: We analyze *F. prausnitzii* phylotypes, butyrate production and epigenetic rgulation in obese, diabetic 2- and lean controls under a four month nutrition intervention.

Methods: We compare fecal and blood samples of the 3 groups at three time points with qPCR and high resolution melt curve analysis (HRMA) and automated ribosomal intergenic spacer analysis (ARISA). *F. prausnitzii* depending butyrate gene levels are detected by qPCR. Epigenetic methylation of CpG sites in promoter regions of FFAR3, TLR2, TLR4, TNF-α and Line1 are investigated with bisulfite-pyrosequencing of the DNA extracted from the blood samples.

Results: Obese- and diabetic groups differ significantly at all-time points compared to controls with fewer *F. prausnitzii* copies. *F. prausnitzii* butyrate levels do not correlate with the total *F. prausnitzii* amount. Analysis of CpGs in the promoter regions of FFAR3, TLR2, and TLR4 showe lower methylation% in obesity and type 2 diabetes. CpG methylation of LINE-1and TNF- show no significant changes between the groups or during the time course. Negative correlation between BMI and methylation of FFAR3 was observed.

Conclusion: Different phylotypes of *F. prausnitzii* and their various functions may lead to differences in the inflammatory genesis in the host. Butyrate producers may have an influence in developing obesity and might also act as starting points for prevention and therapy of obesity.

5th Congress of European Microbiologists FEMS in Leipzig, Germany, July 21 – 25, 2013

12.3. Effects of Antibiotic therapy on the gastrointestinal microbiota and the intervention with L.Casei

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Antibiotic-associated diarrhea (AAD) is discussed to be associated with the growth of pathogens like *C. difficile*, but also a decreased short chain fatty acid fermentation caused by impaired GI microbiota. We compared a therapy with antibiotics

with a combination therapy of antibiotics and *L. casei Shirota* in 340 patients per group. Stool samples from 4 groups/ 56 patients were taken at one time-point before and 2 time-points after antibiotic treatment ± intake of *L. casei*. Fecal samples were investigated for *C. difficile* toxin, changes of bacterial groups and abundance of a crucial enzyme for butyrate production in GI microbiota by qPCR, DGGE, cloning and sequencing using 16S rRNA group specific primers.

Reduced AAD, (18% : 5%) was seen by intervention with *L. casei Shirota*. Antibiotic ± L. casei treated groups showed a decrease of total bacteria, Clostridia clusters IV, XI, XIV, bifidobacteria, the butyryl CoA:acetate CoA-transferase gene and diversity. In the antibiotic group 2 cases of C diff. were detected by toxin specific qPCR as well as a higher abundance of Enterobacteriaceae. In control groups receiving *L. casei* a significant increase of lactobacillus was seen. Combination therapy with antibiotics + *L. casei* resulted in an increase in diversity of two more bands from time point 1 to 3 and a significantly reduced decline in bifidobacteria compared to the antibiotic group. These results indicate the need of a molecular analysis of *C. difficile*. *C. difficile* growth might only contribute to cases of AAD. Probiotic intervention could interfere with the reduced short chain fatty acid metabolism of impaired microbiota in AAD.

4 th ÖGMBT Annual Meeting – Austrian Association of Molecular Life Science and Biotechnoolgy, Graz, September 17 – 19, 2012

12.4. Do changes in GI-microbiota influence epigenetic methylation of inflammatory genes in type 2 diabetes and obesity?

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Obesity and Diabetes mellitus type 2 (DM2) are associated with a systemic low grade inflammation. Changes of the gastro-intestinal (GI) microbiota as well as epigenetic regulation are discussed to trigger this inflammatory response. Different interactions between GI-microbiota and epigenetic mechanisms are discussed. Possible mechanisms are the NF-κB signalling mediated from bacterial cell wall components DNA or the signalling via short chain fatty acids produced by the GI-microbiota.

We enrolled 3 groups of study participants: individuals with (OD) and without (OC) DM2 and lean controls (LC). The OD-participants underwent three months of intervention consisting of nutritional counselling and the glucagon-like peptide-1 agonist Liraglutide. Blood and stool samples were collected at the beginning of the study, after one and after three months. Stool samples were analysed for the abundance and diversity of GI-microbiota using PCR-DGGE, qPCR and 454 Pyrosequencing. Epigenetic methylation of CpG sites in the promoter regions of TLR2, TLR4, FFAR3, TNF-α and Line1 was investigated with bisulfite-pyrosequencing of the DNA extracted from the blood samples.

The diversity and the band pattern of the GI-microbiota were reduced in the OD- and OC-group compared to LC-individuals. The ratio of *Firmicutes*/*Bacteroidetes*, the abundance of lactic acid bacteria subgroups and of *Enterobacteria* increased during the intervention period in OD-participants. In contrast, in OC-individuals with weight loss the ratio of *Firmicutes*/*Bacteroidetes* was decreased.

We saw a decreased CpG methylation in the promoter regions of TLR2, TLR4 and FFAR3 in the OC- and OD-groups compared to LC-individuals. CpG methylation of TNF-α and Line1 did not show significant changes between the groups or during the time course. Further, we observed correlations between anthropometric measurements and methylation of TLR4.

Changes in the GI-microbiota and the DNA-methylation may not only be useful biomarkers but might also act as starting points for prevention and therapy of obesity and DM2.

„Third Clinical Epigenetics International Meeting" – Clinical Epigenetics Society, March 14 and 15, 2013,. Solingen, Deutschland

13. Appendix

13.1. DGGE

Denaturing Gradient Gel electrophoresis is a qualitative Method. A chemical gradient denatures the samples during they move across an acrylamide gel. DGGE can be used to nucleic acids such as DNA and RNA, as well as proteins. DGGE is a genetic profile or “fingerprint”. DGGE offers to separating the DNA fragments by their length and melting behavior. The Gel concentration depends on the GC – content. The polymerization of acrylamide to polyacrylamide is caused by a chain reaction. APS and TEMED are the initiators. APS, as a radical, starts the reaction and TEMED catalyzes the reaction

The first step is DNA extraction, afterwards amplify the DNA (endpoint PCR). PCR – products have to be felling and resuspend. The last step is DGGE.

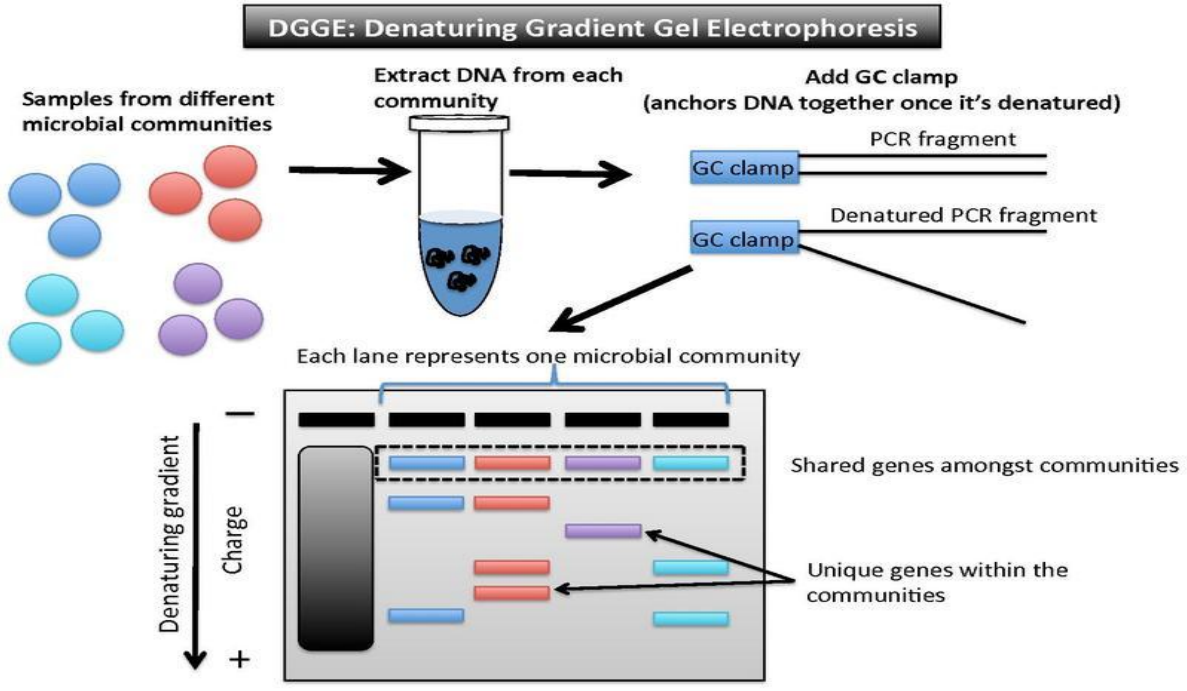

Figure 110: Steps of DGGE

		x samples
Promega MM	50µl	
341 forward GC (50pmol/µl)	1µl	
518 reverse (50pmol/µl)	1µl } MM’	
BSA	2µl	
NFW	41µl	

Table 13: Pipette schema for endpoint PCR for DGGE

Total volume: 5µl template* + 95 µl MM’ = 100µl

*samples (dilute 1:10 from the aliquot), SL (conc.: 10ng/µl) for DGGE, NFW; all in duplicate

SL includes:

1. Bac. thetaiotaomicron DSM 2079
2. Enterococcus faecium
3. C.lept. Koln 16
4. E.coli
5. C.cocc Klon 14
6. L.reuteri
7. Bif.long.ssp.long DSM 20219

95°C	10’	
95°C	1’	
55°C	50’’	x 35 cycles
72°C	50’’	
72°C	5’	

Table 14: Endpoint PCR program for DGGE

Gel - electrophoresis is a method to control the samples/ to separate a mixed population of DNA and RNA fragments by length and to estimate the size of DNA and RNA fragments or to separate proteins by charge. DNA Gel – electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR to control if DNA is in the product. In my case, I used 2% Agarose Gel to control the DNA (5µl of the sample)

Next step is to prepare the samples for DGGE. Pipette 1ml of Ethanol (70%., frozen) and 95µl of PCR sample in a clean tube. Storage over the night at minus 20°C. The next day, centrifuge the samples and throw the hole volume away. The DNA sticks in the tube. For drying the samples, put the tubes on a 30°C heating block. Afterwards, resuspend the samples with 15µl NFW and 5µl loading Part. Now the samples are ready for DGGE.

80% solution	0% solution
40,5g Urea	30ml Acrylamid 40%
48ml Formamid	1,5ml TAE
30ml Acrylamid 40%	➔ fill up to 150ml with dest. H2O
1,5ml TAE	
➔ fill up to 150ml with dest H2O	

Table 15: Register: Preparing DGGE solution

	+	-	0
0% solution			8ml
APS	7ml	7ml	4ml
TEMED	50ml	50ml	35ml

Table 16: APS + TEMED for preparing DGGE Gel

Gradient for all bacteria: 30% - 62,5%

	+	-
80%	6,75ml	3,75ml
0%	2,18ml	7,80ml

Table 17: Solution for gradient

Pipette 20µl of the samples in the DGGE slots. For this kind of work you use the Hamilton pipette.

Temperature of the DGGE Gel: 60°C

Volt: 175, Duration: 6 hours

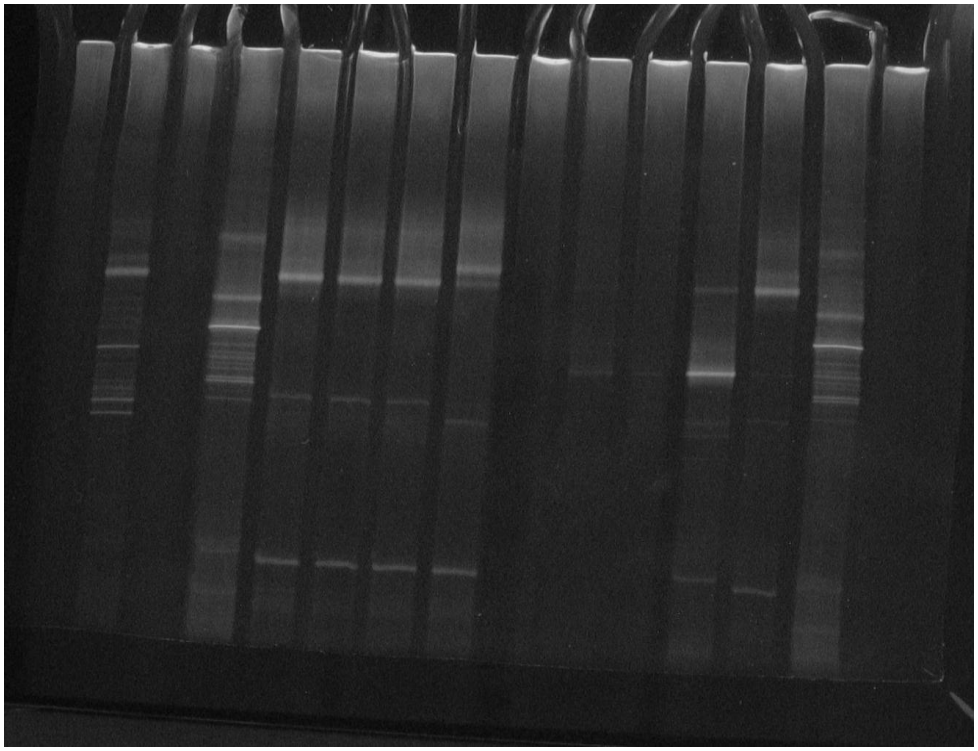

Figure 121: Picture of a DGGE – Gel

For cloning, select the PCR or DGGE sample with the exciting band. A DNA Purification is necessary to purify the DNA.

13.2. QIAquick PCR Purification Kit Protocol

Protocol from the QIAquick Spin Handbook 2008:

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.

(add 500µl of Buffer PB to 100µl PCR sample)

2. Place a QIAquick spin column in a provided 2ml collection tube.

3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 45''.

4. Discard flow – through. Place the QIAquick column back into the same tube.

5. To wash, add 750µl Buffer PE to the QIAquick column and centrifuge for 45''.

6. Discard flow – through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1min.

7. Place QIAquick column in a clean 1,5ml tube.

8. To elute DNA, add 30µl elution buffer (Buffer EB) to the center of the QI-Aquick membrane, let the column stand for 1min, and then centrifuge.

9. If the purified DNA is to be analyzed on a gel, add 1ml volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel. (negative control: Buffer EB)

13.3. Cloning

- Preparing Agar plates (liquid and fixed one) – LB media
- in the fixed solution, after preparing a control plate, add 1 tube AMP in 500ml solution
- spread out X-Gal on the plates
- storage in the fridge

LB – Medium: (lysogeny broth – Media)

- nutritionally rich medium – used for the growth of bacteria
- industry standard for the cultivation of E. coli
- mainly used in molecular microbiology applications for the preparation of plasmid DNA and recombinant proteins

Ingredients:

- several common formulations of LB -> they generally share a similiar composition of ingredients used to promote growth
- The formulations generally differ in the amount of sodium chloride, thus providing selection of the appropriate osmotic conditions for the particular bacterial strain and desired culture conditions. The low salt formulations, Lennox and Luria, are ideal for cultures requiring salt-sensitive antibiotics.
- Peptides and peptones are provided by tryptone. Trypton is produced by pancreatic digestion of casein (so you can use also pepton of casein instead of Trypton).
- Vitamins and certain trace elements are provided by yeast extract.

- Sodium ions for transport and osmotic balance are provided by sodium chloride.

Ligation: (promega pGEM T easy Vector System I)
Ligation with purified template and control – control is included in the Kit

7,5 µl Buffer
5,5 µl PCR sample or 3µl control
1 µl Vector
1 µl Ligase
Total volume: 15µl

Table 18: Ligation

- Incubate over the night at 4°C

Transformation: (One Shot Top 10 cells – Invitrogen)

1. 5µl of the ligation are transferred to the competent cells. (they are taken from minus 80°C)

2. 30 minutes incubate on ice

3. Heat shocked for 30 seconds at 42°C on a thermobloc and immediately put on ice for additional two minutes

4. Add 250µl of pre-warmed LB-medium solution to each transformation reaction and the tubes are incubated at 37°C for one hour at 225 – 330rpm in a shaking incubator

5. The transformation reaction are platted on plates, preheated to 37°C:

- ✓ 30µl, 50µl and 100µl are platted on the plates

6. Incubate over night

7. The next day, the white colonies are harvested and resuspended in a tube containing 40µl TE buffer.

8. Afterwards the white colonies are platted again on the agar plates.

9. Incubate over night at 37°C again. The next day, you pick the line number three to get clean colonies; solute again in 40µl TE buffer (these kinds of picked colonies you need for the PCR and sequencing)

10. Storage the tubes at minus 20°C, the agar plates in the fridge at 4°C

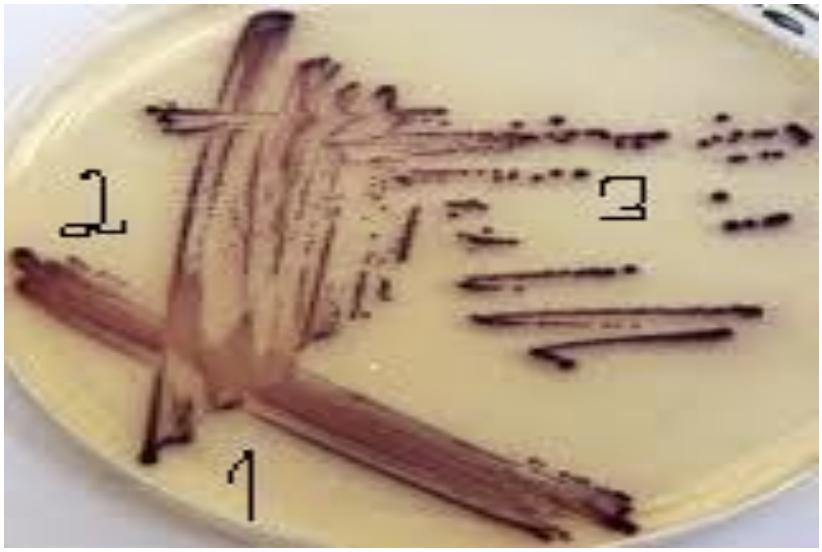

Figure 132: Spread out the second picked colonies

13.4. Sequencing

		x samples
Promega MM	12,5µl	
T7 (conc.: 100 pmol/µl)	0,1875µl	
SP6 (conc.: 100pmol/µl)	0,1875µl } MM'	
BSA	0,5µl	
NFW	10,125µl	

Table 19: PCR program after cloning for Sequencing

Total volume: 1,5µl template + 23,5µl MM' = 25µl

Condition for Sequencing:

200bp to 500bp – 10 ng/µl

500bp to 1000bp – 20 ng/µl

➔ Picodrop

Volume: 15µl/run

The samples are sequenced by Sanger Sequencing (LBC lab). We compared the results with the http://rdp.cme.msu.edu/ and with the http://blast.ncbi.nlm.nih.gov/. These sequenced samples are used for the standard control at qPCR.

Vector (T7, SP6):

5' TGTAA TACGA CTCAC TATAG GGCGA ATTGG GCCCG ACGTC GCATG CTCCC GGCCG CCATGGCGGC CGCGG GAATT CGATT 3'

5' ATCAC TAGTG AATTC GCGGC CGCCT GCAGG TCGACCATAT GGGA GAGCT CCCAA CGCGT TGGAT GCATA GCTTG AGTAT TCTAT AGTGT CACCT AAAT 3′

Prev:

GCCGCCATGGCGGCCGCGGGAATTCGATTCACCAAGGCGACGATCAG-TAGGGGTTCTGAGAGGAAGGTCCCCCACATTGGAACTGAGACAC-GGTCCAAACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGACGA-GAGTCTGAACCAGCCAAGTAGCGTGCAGGATGACGGCCCTATGGGTT-GTAAACTGCTTTTATAAGGGAATAAAGTGAGTCTCGTGAGACTTTTT-GCATGTACCTTATGAATAAGGACCGGCTAATTCCGTGCCAGCAGCCGCGG-TAATACGGAAGGTCCGGGCGTTATCCAATCACTAG-TGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAAC-GCGTTGGATGCATAGCTTGAGTATTCT

classifier_seq Root[100%] Bacteria[100%] "Bacteroidetes"[100%] "Bacteroidia"[100%] "Bacteroidales"[100%] "Prevotellaceae"[100%] Prevotella[100%]

Akk:

GCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTCAGCAC-
GTGAAGGTGGGGACTCTGGCGAGACTGCCCAGATCAACTGGGAG-
GAAGGTGGGGACGACGTCAGGTCAGTATGGCCCTTATGCCCAGGGCTG-
CACACGTACTACAATGCCCAGTACAGAGGGGGCCGAAGCCGCGAGGCG-
GAGGAAATCCTAAAAACTGGGCCCAGTTCGGACTGTAGGCTGCAACCCGCC-
TACACGAAGCCGGAATCGCTAGTAATGGCGCATCAGCTACGGCGCCGTGAA-
TACGTTCCCGGGTCTTGTACACACCGCCCGTCACATCATGGAAGCCGGTCG-
CACCCGAAGTATCTGAAGCCAACCGCAAGGAATCACTAG-
TGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAAC

GCGTTGGATGCATAGCTTGAGTATTCT

classifier_seq Root[100%] Bacteria[100%] "Verrucomicrobia"[100%] Verrucomicrobiae[100%] Verrucomicrobiales[100%] Verrucomicrobiaceae[100%] Akkermansia[100%]

F.prausnitzii:

GCCATGGCGGCCGCGGGAATTCGATTGGAGGAAGAAGGTCTTCG-
GATTGTAAACTCCTGTTGTTGAGGAAGATAATGACGG-
TACTCAACAAGGAAGTGACGGCTAACTACGTGCCAGCAGCCGCGGTAAAAC-
GTAGGTCACAAGCGTTGTCCGGAATTACTGGGTGTAAAGGGAGCG-
CAGGCGGGAAGATAAGTT-
GGAAGTGAAATCCATGGGCTCAACCCATGAACTGCTTTCAAAACTGTTTTTCT
TGAGTAGTGCAGAGGTAGGCGGAATTAATCACTAG-
TGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAAC-
GCGTTGGATGCATAGCTTGAGTATTCTA

classifier_seq Root[100%] Bacteria[100%] Firmicutes[100%] Clostridia[100%] Clostridiales[100%] Ruminococcaceae[100%] Faecalibacterium[100%]

14. Curriculum Vitae

Simone Dworzak

EDUCATION AND QUALIFICATIONS:

since 2011	University of Vienna Master student of nutritional science focus: molecular nutrition Master thesis: “Gastrointestinal microbiota in type 2 diabetes”
2010 – 2011	Medical council of Vienna Training of state inspected and certified ordination assistant Graduation: May 2011
2005 - 2011	University of Vienna Bachelor student of nutritional science Bachelor thesis: “Celiac disease and osteoporosis” Graduation: April 2011: Bakk.rer.nat.
2004 – 2005	University of Vienna Study of pharmacy

2000 – 2004	Erzbischöfliches Real- und Aufbaugymnasium Hollabrunn focus of this school: human biology and human psychology Graduation: June 2004: Matura

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WORK EXPERIENCE:

since 2013	Health Bio Care, Vienna scientific assistant: feces analyzes and evaluation
since 2012	University of Vienna student assistant: Tutorium: Übungen zu mikrobiologischen und molekularbiologischen Fragen der Lebensmittel
since 2006	pediatrician office, Vienna Gruppenpraxis DDr.Voitl und Partner Facharzt für Kinder- und Jugendheilkunde ordination assistant
since 2004	Dorfheurigen Dworzak, Deinzendorf waitress and kitchen assistant

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PRACTICAL TRAINING:

March 2013	University of Vienna working group: Dr. Alexander Halsberger focus: microbiology and epigenetic
October 2012 – March 2013	University of Vienna working group: Dr. Karl – Heinz Wagner study: “Active Aging” assistance: interview, mini mental test
Summer 2008	Stamag Malzfabrik Stadlau, Vienna laboratory: quality control
2001 – 2006	Espresso und Bäckerei Blei, Retz waitress

PUBLICATIONS:

Paper:

Abundance and diversity of microbiota in type 2 diabetes and obesity

Remely Marlene, Dworzak Simone, Hippe Berit, Zwielehner Jutta, Aumueller Eva, Brath Helmut, Haslberger Alexander

Paper: Journal of Diabetes and Metabolism, 2013

Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity

Marlene Remely, Christine Merold, Simone Dworzak, Eva Aumüller, Berit Hippe, Julia Zanner, Helmut Brath, Alexander G. Haslberger

Paper: PLOS ONE; submitted, 2013

Abstract and Poster:

Effects of antibiotic therapy on the gastrointestinal microbiota and the intervention with L. casei

Simone Dworzak, Marlene Remely, Christoph Kamhuber, Felix Stockenhuber, Alexander Haslberger

4. ÖGMBT Jahrestagung; September 17th-19th 2012; Graz, Austria

Abstract and Poster

Microbiota and epigenetic regulation of inflammatory mediators in metabolic syndrome and caloric restriction

Remely M, Aumueller E, Chen Y, Dworzak S, Haslberger A

3rd International Workshop Gut microbiota in Health and Disease, Sepember 13th - 15th, Maastricht, Netherlands

Abstract and 2nd Posterprice

Relation of *Faecalibacterium prausnitzii* phylotypes and epigenetic regulation in obese, diabetic patients and controls

Berit Hippe, Simone Dworzak, Marlene Remely, Eva Aumüller, Christine Merold, Alexander G Haslberger

5th Congress of European Microbiologists FEMS in Leipzig, Germany, July 21 – 25, 2013

Abstract and Poster

Do changes in GI-microbiota influence epigenetic methylation of inflammatory genes in type 2 diabetes and obesity?

Marlene Remely, Christine Merold, Ya Chen, Eva Aumüller, Daniela Jahn, Simone Dworzak, Berit Hippe, Helmut Brath, Alexander G. Haslberger

„Third Clinical Epigenetics International Meeting" – Clinical Epigenetics Society, March 14 and 15, 2013, Solingen, Deutschland

Abstract and Poster

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