Abundance and Diversity of Microbiota in Type 2 Diabetes and Obesity

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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. 

Whether differences seen in the abundance of certain groups and in the 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; DSMZ: German collection of Microorganisms and Cell Cultures; FFQ: The Austrian Research Promotion Agency; 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; HbA: 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: Peptide Activator Inhibitor-1; qPCR: Quantitative Polymerase Chain Reaction; rDNA: Ribosomal DNA; SCFA: Short Chain Fatty Acid; T: Time Point; TLR: Toll Like Receptor; TNFa: Tumor Necrosis Factor Alpha; UPGMA: Unweighted Pair Group Method with Arithmetic Mean; UK: United Kingdom

Introduction

The gut microbiota an integral part of a complex network coordinating the physical and chemical elements of the intestinal barrier together with the immune, sensory, neuromotor- and enteroeendocrine 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 specialized 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 or after conventionalization 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 reversed 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 Bacteriobes is increased in diabetic mice models and humans. Relative abundances of Actinobacteria and Verrucomicrobia do not differ between diabetic and non-diabetic individuals. Bacterioideae-Prevotella versus C. coccoides - E. rectale groups are positively associated with plasma glucose, but negatively correlated with BMI [13]. Wu et al. [12] 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 Bacterioidetes 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 significantly 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, Liraglutide 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 years) were enrolled in cooperation with a Diabetes Outpatient Clinic. In addition we enrolled fourteen obese participants with no established insulin resistance (aged 38 ± 14 years) and nineteen healthy lean controls (aged 30 ± 8 years) (Table 1). Obese and type 2 diabetics received an intervention of nutritional counseling, type 2 diabetics an additional therapy with GLP-1-Agonists (Liraglutide). 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 Fast Prep™ Lysing Matrix E tubes (MP Biodiagnostics, 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 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, USA) following the instructions of the manufacturer. Clone libraries were screened according to Schabereiter-Gurtner et al. [22]. The obtained nucleotide sequences from Sanger sequencing (LGC Genomics, Germany) 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, Australia) using 16S rDNA group specific primers (Tables 2 and 3). Specificity was checked with the Probe Match 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 Pirikera et al. [23].

| Group | characterization | rate | BMI (± SD) [kg/m²] |
|-------|-----------------|------|-------------------|
| 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 1: Characterization of participant groups.
PCR/DGGE

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

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 blautiacoccoides DSM 935, Clostridium leptum DSM 753, Bifido bacterium longum sp. longum DSM 20219 and Bacteroides thetaiotaomicron DSM 2079.

High throughput sequencing

Sixteen samples were analyzed with Roche GS FLX+ Titanium next generation sequencing (ILGC sequencing GmbH, Germany). 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 tests, 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, USA. P values < 0.05 were determined as statistically significant. DGGE-gels were evaluated with Gel compare II (Applied Maths, Belgium).

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 PCR-DGGE 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, Archea were significantly (p<0.05) more frequent in the type 2 diabetics (18 ± 5 patients) compared to control groups, followed by obese patients with (4 ± 0 participants) and lean controls (5 ± 3 participants).

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
44.0% of lean controls did sports 1-3 times per week. HbA and type 2 diabetics (64.0% T1, 52.0% T3 never do sports), whereas 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 compared 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. 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 species.
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. 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. 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 Verrucomicrobia, PC2 Proteobacteria, Actinobacteria and Bacteroidetes (Figure 4), Larsen et al. shows also a higher separation between the groups attributed to Bacteroides, Firmicutes and Verrucomicrobia (45%) and in second direction the combination of Proteobacteria and Actinobacteria (28%) [13].

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 Firmicutes 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%; Figures 2,3). Intervention here resulted in a weight loss of (4.87 ± 3.68 kg). Weight loss was highly diverse between individuals.

Consequences of these shifts remain under discussion for their effects on endotoxin mediated low grade inflammation, but also for consequences on energy extraction 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]. Schwierz 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 GLP-1-Agonist 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, Strep-tooccus 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].

| 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; D: Type 2 Diabetics; T: Time Point

Table 4: Number of bands observed in PCR-DGGE fingerprinting in lean controls, obese patients and type 2 diabetes at all three time points.
Caloric restriction increases abundance of *Akkermansia* and *Archaea*?

We report an enhanced abundance of *Akkermansia* (Figures 2 and 3) and a higher appearance of *Archae* 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 anorexie 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 H$_2$ by the synthrophic relationship with bacteria improves fermentation efficiency [7,41]. Moreover less energy has been measured in obese feces in mice relative to lean littersmates [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 in the development of obesity and type 2 diabetes.

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