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ARTICLE

Perturbation of the mucosa-associated anaerobic gut microbiota in streptozotocin-induced diabetic rats

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ABSTRACT Our aim was to map the gut region-specific differences of the mucosa-associated microbiome distribution in a streptozotocin-induced diabetic rat model. Tissue samples from the duodenum, ileum and colon were collected 10 weeks after the onset of hyperglycaemia to analyse the mucosa-associated microbiota using next-generation DNA sequencing. Striking differences were observed in the mucosa-associated microbiota of the duodenum between diabetic and control rats. A significant invasion of the aerobic genus *Mycoplasma* was apparent in diabetes, and the abundance of the anaerobic phylum Firmicutes decreased massively. It is noteworthy that insulin treatment eliminated the *Mycoplasma* invasion in the duodenum and apparently restored the anaerobic environment in the mucosa. In the ileum the abundance of the phylum Firmicutes increased in the diabetic samples. Although the proportion of the phylum Proteobacteria decreased moderately, its composition changed significantly, and insulin treatment induced only minor alterations. In the diabetic samples of colon, the abundance of the phylum Firmicutes decreased slightly, the relative number of the bacteria in the phylum Bacteroidetes increased strongly as compared to the control values, and after insulin treatment this increase was more significant. Chronic hyperglycaemia has the most prominent effect on the mucosa-associated microbiota in the duodenum.
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Introduction

Type 1 diabetes (T1D) is an autoimmune disease with a strong genetic basis, and it is obvious that several environmental factors contribute to the disease (Jerram and Leslie 2017; Kugelberg 2017). The gastrointestinal tract is colonized by microbiota, a large and complex microbial community, consist of mainly bacteria. Microbiota also includes commensal populations of fungi, viruses, archaea, and protists (de Oliveira et al. 2017; Flemer et al. 2017; Matijašić et al. 2020). The role of the gut microbiota in T1D ethology has been the subject of research over the last decade to clarify disease development and to determine preventive approaches (e.g., diet manipulation, probiotic administration) (de Oliveira et al. 2017; Marino et al. 2017; Mishra et al. 2019; Tanca et al. 2018; Tian et al. 2017; Wirth et al. 2014).

Studies corroborated that intestinal dysbiosis affects gut permeability via their metabolites and plays a role in T1D development, but there is no evidence for the specific role of intestinal microbiota in the development of autoimmunity to beta-cells and in tissue damage (de Oliveira et al. 2017; Knip and Honkanen 2017; Paun et al. 2017; Tian et al. 2017).

Investigations along the longitudinal axis of the gastrointestinal (GI) tract corroborated a high level of similarity between the rat model and humans in the oxygenation levels as well as in microbial diversity (Li et al. 2017). Oxygen levels in the air (≈21% = 145 mmHg = 19,331.7 Pa) drop dramatically to around 32 mmHg (4,266.3 Pa) in the rat and human duodenum and <3 mmHg (<400.0 Pa) in the colon (Albenberg et al. 2014). Due to the radial gradients the oxygenation landscape in the GI tract is inhomogeneous but with all practical measures it is considered an anaerobic environment inhabited by facultative and obligate anaerobic microbial communities. A correlation between the diminishing oxygen levels and

the characteristic anaerobic microbes exists along the GI tract (Albenberg et al. 2014; Wirth et al. 2014). Previous studies have indicated the involvement of perturbed microbiota in inflammatory bowel diseases (IBD), i.e. Crohn' disease and ulcerative colitis (Chermesh and Shamir 2009). Although there are numerous similarities between IBD and T1D, the microbial alterations caused by T1D in the mucosa have not been addressed before.

The host-microbiota interactions have been recognized as highly site-specific, and the local crosstalk determines intestinal function and physiology (Sommer and Backhed 2016). The overwhelming majority of the samples used to study microbial events in the gut were taken from colonic stools (Flemer et al. 2017; Hu et al. 2017; Marino et al. 2017; Paun et al. 2017; Qi et al. 2016) with the assumption that the microbiota of the colon represents the microbiota of the entire GI tract. Although the ease of sampling may justify the selection of this sampling site, numerous studies warned that the microbiome of the colon does not unequivocally represent the microbial activities in the upper GI regions. Our previous experiments investigated the possible correlation among the diabetes-related gut region-dependent nitrergic myenteric neuropathy, the altered mesenteric capillaries (Bodi et al. 2012; Izbeki et al. 2008) and the spatially restricted distribution of the lumen-associated microbiota (LAM) in the GI tract (Wirth et al. 2014). The regionally distinct alterations in the microbiome along the longitudinal axis of the GI tract correlated well with the regional manifestations of diabetes-related enteric neuropathy and mesenteric capillary damage (Wirth et al. 2014).

In this study, we investigated the composition of the mucosa-associated microbiome (MAM) along the gut in the same STZ-induced diabetic and insulin-treated diabetic rat model to search for a causal relationship between the prevalence of bacteria in the specific parts of the GI tract and region-specific myenteric nitrergic neuropathy. We noted that surprisingly a significant perturbation of the anaerobic microbial community took place in the T1D duodenum, which disappeared upon insulin treatment although insulin could not restore the healthy GI microbiome.

Materials and Methods

Animal model

The experiments were performed on male Wistar rats (Crl: WI BR; Toxi-Coop Zrt., weighing 210-260 g) with strict adherence to the National Institutes of Health (Bethesda, MD, USA) guidelines and the EU directive 2010/63/EU for the protection of animals used for scientific purposes. The study was approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority), with the license number XX./1487/2014. The rats were kept in the animal care facility of the Institute of Surgical Research of the University of Szeged in Type III plastic cages in a 12/12-h day/night cycle under standard air temperature and humidity conditions. Standard laboratory chow (Farmer-Mix Kft., Zsámbék, Hungary) and water were provided ad libitum.

The rats were divided randomly into three groups: STZ-induced diabetics (n = 3), insulin-treated diabetics (n = 4), and sex- and age-matched controls (n = 5). Hyperglycaemia was induced as described previously (Bodi et al. 2012; Izbeki et al. 2008). The animals were considered diabetic if the non-fasting blood glucose concentration was higher than 18 mM. From this time on one group of hyperglycaemic rats received a subcutaneous injection of insulin (Humulin M3; Eli Lilly Nederland, Utrecht) each morning (2 IU) and afternoon (2 IU). The non-fasting blood glucose concentration and the weight of each animal were measured weekly.

Tissue handling

Ten weeks after the onset of hyperglycaemia, the animals were sacrificed by cervical dislocation under chloral hydrate anaesthesia (375 mg/kg i. p.). The pancreas and gut segments of the control, STZ-induced diabetic and insulin-treated diabetic rats were dissected and rinsed in sterile distilled water (Milli-Q).

Immunohistochemical staining

For immunohistochemistry, paraffin-embedded pancreas sections were immunostained with insulin marker. Briefly, after blocking in PB containing 0.1% bovine serum albumin, 10% normal goat serum and 0.3% Triton X-100, the samples were incubated overnight with anti-insulin (mouse; Sigma-Aldrich, Budapest, Hungary; final dilution 1:100) primary antibody. After washing in PB, sections were incubated with anti-mouse Cy3 (Jackson Immuno Research Laboratories, Baltimore Pike, PA; final dilution 1:200) secondary antibody for 2 hours. All incubations were carried out at room temperature. Negative controls were performed by omitting the primary antibody, when no immunoreactivity was observed. Sections were mounted on slides in Fluoroshield™ with DAPI histology mounting medium (Sigma-Aldrich, Budapest, Hungary), observed and photographed with a Zeiss Imager Z.2 fluorescent microscope equipped with an Axiocam 506 mono camera.

DNA isolation and next-generation sequencing

Three-cm-long samples were taken from the duodenum (1 cm distal to the pylorus), the ileum (1 cm proximal to the ileocaecal junction), and the proximal colon and processed

Table 1. Weight and glycaemic characteristics of the three experimental groups of rats (means ± SEM).

	Weight (g)		Blood glucose level (mM)	
	initial	final	initial	final (average)
Controls (n = 5)	232.2 ± 7.29	486 ± 4.93 *	7.08 ± 0.22	6.3 ± 0.13
STZ-induced diabetics (n = 3)	235.3 ± 10.48	382.7 ± 3.53 *, °	6.6 ± 0.1	23.31 ± 0.53 *, °
Insulin-treated diabetics (n = 4)	251.5 ± 4.35	481.5 ± 13.4 *, +	6.65 ± 0.18	9.48± 0.14 *, °, +

*: Initial vs. final ($p < 0.0001$); °: control vs. diabetic group ($p < 0.0001$); +: diabetic vs. insulin-treated diabetic group ($p < 0.0001$). Statistical analysis was performed with one-way ANOVA and the Newman–Keuls test. Analyses were carried out with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, the United States of America). A probability of $P < 0.05$ was set as the level of significance.

for metagenomic studies. For removing the intestinal contents, the dissected gut segments were washed thoroughly twice with a strong jet of sterile distilled water (2 x 10 ml, Milli-Q), and the 3-cm-long intestinal tissue samples were placed and shaken in 10 ml sterile distilled water (Milli-Q) in sterile Falcon tubes.

Three ml of supernatant was used for total-community DNA isolation. The extractions were carried out with the Macherey-Nagel Nucleospin Soil kit following the supplier's instructions (Macherey-Nagel: 740780.250, Germany, Düren). For efficient cell lysis, SL1 buffer, SLX Lysis Enhancer and deadbeat were used. The samples originating from the same animal group and the same gut segments were pooled. Shotgun metagenome sequencing was carried out following the recommendations of the Ion Torrent PGM sequencing platform (Life Technologies, Hungary, Budapest). The preparation of sample libraries was made by the Life Technologies IonXpress fragment plus library protocol (4471269). Samples were quantified by using the Ion device library quantitation kit (4468802) and Step One Real Time PCR (Applied Biosystems Hungary, Budapest). Ion OneTouch 2 and Ion OneTouch ES devices were used with the Ion PGM Template OT2 200 kit (4480974). Barcoding was made by the IonXpress barcode kit (4471250). Sequencing was performed using the Ion PGM Sequencing kit (4474004) on an Ion Torrent PGM 316Chip (Wirth et al. 2014). Quality values were determined for each nucleotide. The 100-200 nt individual reads were further analysed using the MG-RAST software package, which is a modified version of RAST (Rapid Annotations based on Subsystem Technology). The MG-RAST pipeline performs quality control, protein prediction, clustering and similarity-based annotation on nucleic acid sequence datasets. The MG-RAST server analyses against several reference datasets (protein and nucleic acid databases) (Meyer et al. 2008). The acceptable percentage of identity was set to >80%, the minimum alignment length was 50 nt and the e-value cut-off was <10-5. The generated matches of external databases were used to compute the derived data (Randle-Boggis et al. 2016; Wirth et al. 2014). Eukaryotic and unassigned data were disregarded during the distribution calculation of taxonomical abundance. Data are available under the „Microbiome of rat intestinal epithelium" project in MG-RAST.

Results

Characteristics of experimental animals

The general characteristics of the control, diabetic, and insulin-treated diabetic rats 10 weeks after the onset of hyperglycaemia are shown in Table 1. The diabetic rats had reduced body weight and an increased blood glucose concentration (23.31 ± 0.53 mM) as compared with the age- and sex-matched controls (6.30 ± 0.13 mM). The insulin-treated diabetic rats did not differ significantly from the control animals in this respect. Their average blood glucose concentration was significantly elevated (9.48 ± 0.14 mM), but it was closer to the control level.

Anti-insulin immunohistochemical staining showed smaller Langerhans islets and weaker insulin-immunostaining in the diabetic and insulin-treated diabetic animals than in the controls (Fig. 1).

The composition of the mucosal microbiome along the rat GI tract

Two sets of metagenomic data were evaluated in the present study. The distributions were determined at phylum, class and order levels (Fig. 2-4). Eukaryotic and unclassified data were disregarded. In this way, we present the distribution of abundances of the bacterial taxa longitudinally along the diabetic intestinal mucosa for the first time.

The mucosa-associated microbiome of the diabetic duodenum

Striking differences were observed in the composition of the anaerobic microbial community of the duodenum between the diabetic and the control rats. Within the domain Bacteria, the phylum Firmicutes (69%) predominated in the control duodenum. The majority of the Firmicutes were

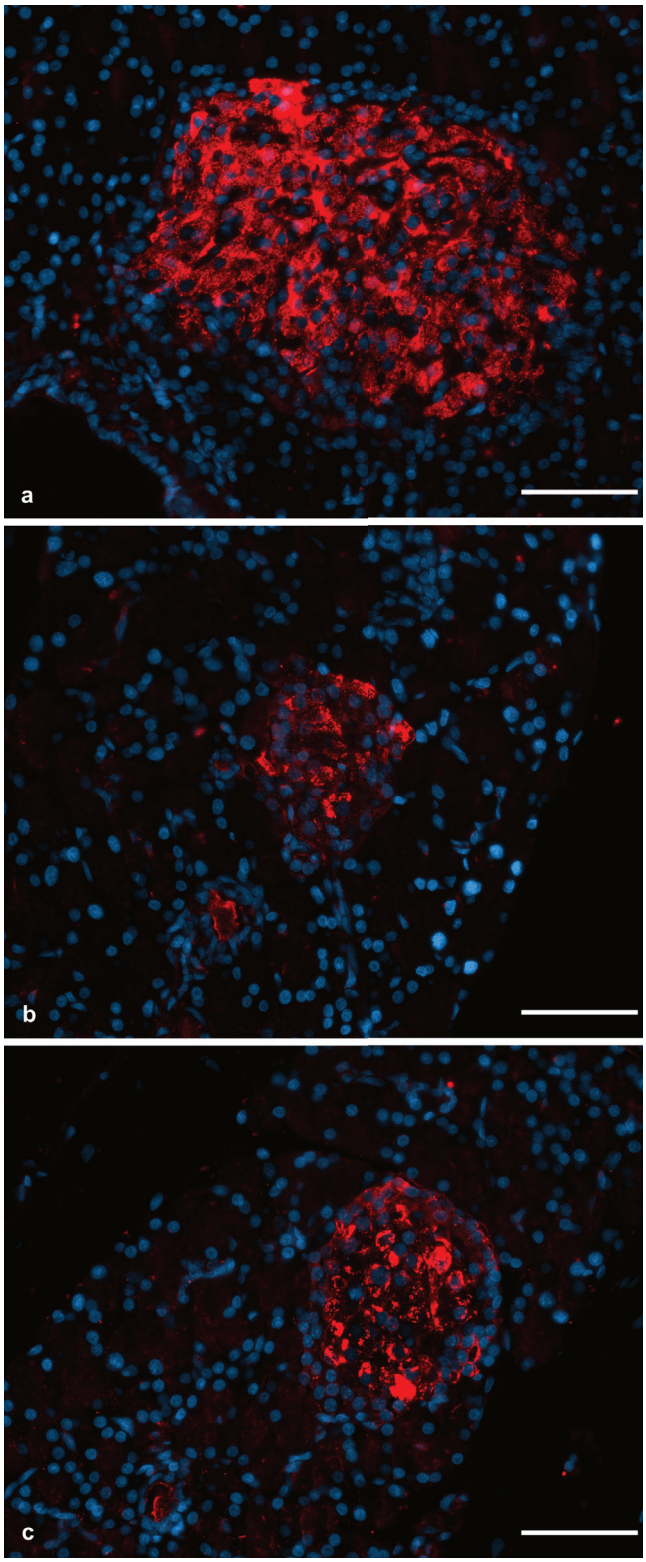

Figure 1. Insulin immunoreactivity in paraffin sections from control (a), diabetic (b) and insulin-treated diabetic (c) rat pancreas (red: insulin; blue: DAPI). Scale bar: 40 µm.

identified to the classes Clostridia (66%) and Bacilli (2%). A substantial invasion of the aerobic genus Mycoplasma of the phylum Tenericutes (not observed in the controls) was apparent in diabetes (14%), and the abundance of the phylum Firmicutes decreased massively (about 56% vs. 69% in controls). The class Bacilli, including the order Lactobacillales was not observed in the diabetic samples (Fig. 2).

The phylum Proteobacteria showed decreased abundance in diabetes as compared to the controls, and within this phylum a remarkable change was observed at the level of lower taxonomic units. The abundances of the classes Gammaproteobacteria and Betaproteobacteria decreased, whereas the class Deltaproteobacteria (not observed in the controls) appeared (2%).

From among the other phyla, the phylum Actinobacteria was appreciably represented both in the control (4%) and in the diabetic (3%) duodenum.

It is noteworthy that insulin treatment eliminated the Mycoplasma (1%) infiltration in the duodenum (Fig. 2). In other aspects, the diversity and distribution of prokaryotes in the insulin-treated diabetic rats did not differ markedly from the healthy controls. The majority of the domain Bacteria was identified in the phyla Firmicutes (65%) and Proteobacteria (~ 25%). The phylum Bacteroidetes (4%) was also represented in the insulin-treated duodenum. As in the control duodenum, the order Lactobacillales was also observed in the insulin-treated samples (3%).

The mucosa-associated microbiome of the diabetic ileum

The majority of the domain Bacteria belonged to the phyla Firmicutes (63%) and Proteobacteria (~25%) in the control ileum. The abundance of the phylum Firmicutes increased in the diabetic samples (68%) (Fig. 3).

Although the proportion of the phylum Proteobacteria decreased moderately (22%), its composition changed strikingly. The class Epsilonproteobacteria, including the order Campylobacterales was basically eradicated from the diabetic samples, whereas in the control it represented 10% of the Bacteria.

The diversity and distribution of prokaryotes in the insulin-treated diabetic rats did not differ markedly from the healthy controls (Fig. 3). The abundances of the predominant phyla Firmicutes and Proteobacteria were about 62% and 26%, respectively. The class Epsilonproteobacteria, including the order Campylobacterales (9%), was also detected in the insulin-treated samples.

The mucosa-associated microbiome of the diabetic colon

The phyla Firmicutes, Bacteroidetes and Proteobacteria still ruled over the microbial landscape, totaling about

Figure 2. Compositions of the control (a), diabetic (b) and insulin-treated diabetic (c) mucosa-associated microbiome in the duodenum at phylum, class and order levels. The abbreviated and color-coded taxa are indicated on the right side in taxonomic levels. A striking invasion of the genus *Mycoplasma* was apparent in diabetes and the representation of the phylum Firmicutes decreased massively. It is noteworthy that the insulin treatment eliminated the *Mycoplasma* invasion.

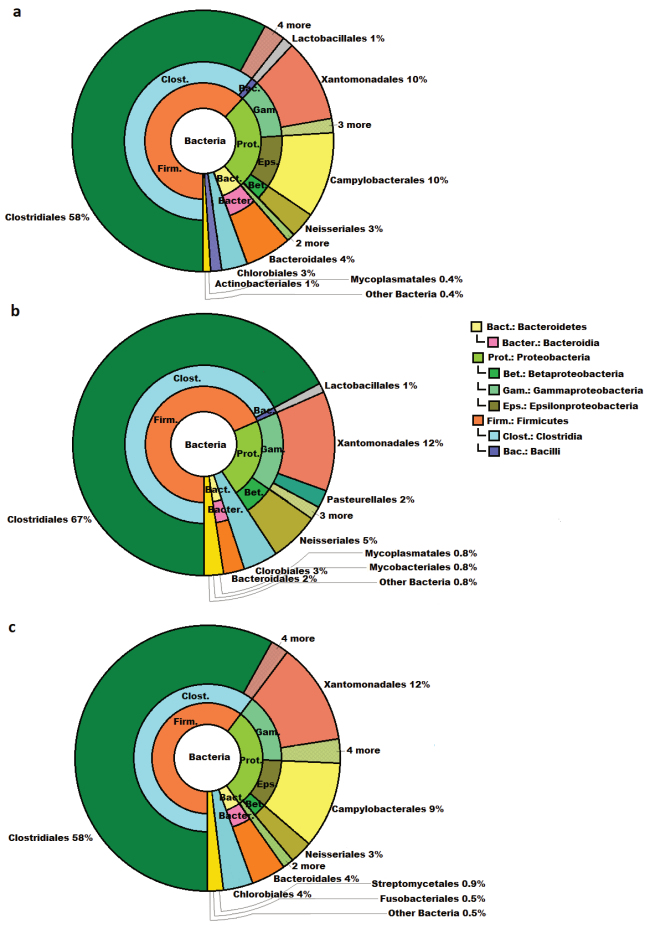

Figure 3. Compositions of the control (a), diabetic (b) and insulin-treated diabetic (c) mucosa-associated microbiome in the ileum at phylum, class and order levels. The abbreviated and color-coded taxa are indicated on the right side in taxonomic levels. In the domain Bacteria the phyla Firmicutes and Proteobacteria predominated. The composition of the phyla Proteobacteria changed strikingly. The class Epsilonproteobacteria was basically eradicated from the diabetic samples.

39%, 31% and 17% of the population in the control colon, respectively. In the diabetic samples the abundance of the phylum Firmicutes decreased slightly (~35%); the relative number of the bacteria belonging to the phylum Bacteroidetes increased strongly (44%) as compared to the control values (Fig. 4).

The abundance of the phylum Proteobacteria (6%) was almost three times lower in the diabetic colon relative to the control. Both Epsilonproteobacteria and Gammaproteobacteria showed decreased abundance in the diabetic colon, moreover the order Campylobacterales was almost eradicated (1% vs. 8% in the control).

The phyla Firmicutes (49%) and Bacteroidetes (26%) accounted for the overwhelming majority of bacteria present in this gut segment of insulin-treated rats (Fig. 4), followed by the Proteobacteria (19%).

The abundance of the phylum Firmicutes was notably higher (49%) in the insulin-treated samples as compared to the controls (39%). In this phylum the proportion of the unclassified Clostridiales showed a major increase relative to the controls (28% vs. 15% in the control).

The abundances of the classes Epsilonproteobacteria (~7%), Gammaproteobacteria (9%) and the order Campylobacterales (7%) were similar to the corresponding control data.

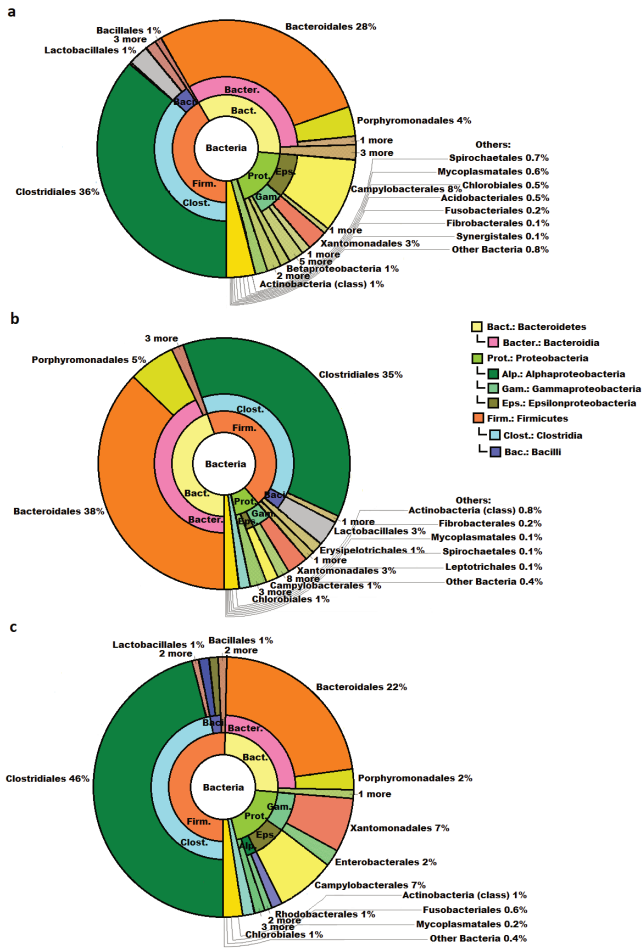

Figure 4. Compositions of the control (a), diabetic (b) and insulin-treated diabetic (c) mucosa-associated microbiome in the colon at phylum, class and order levels. The abbreviated and color-coded taxa are indicated on the right side in taxonomic levels. The domain Bacteria comprised the phyla Firmicutes, Bacteroidetes and Proteobacteria. The phylum Bacteroidetes increased, whereas the phylum Proteobacteria decreased in abundance in the diabetic colon. In the insulin-treated colon the abundance of the phylum Firmicutes was notably higher than in the controls.

Discussion

A growing number of evidences support site-specific host-microbiota interactions and local host-microbiota crosstalk determining intestinal functions and physiology (Bashir et al. 2016; Kelly et al. 2017; Sommer and Backhed 2016; Wang et al. 2010; Wirth et al. 2014). Our previous results (Wirth et al. 2014) showed strong correlation between the regionally distinct alterations of LAM in STZ-treated rats with the regional manifestations of diabetes-related enteric neuropathy and mesenteric capillary damage (Bodi et al. 2012; Izbeki et al. 2008). These data suggested that the myenteric neurons in a specific gut segment are not only targets of T1D, but a significant factor in the pathogenesis of autoimmune diabetes and enteric neuropathy initiated by the gut region-specific alteration of the LAM. The massive invasion of the Gram-negative *Klebsiella* in the ileum could be directly associated with the inflammation, and it could be a critical environmental trigger, initiating the pathological cascade from the epithelium to the enteric neurons, resulting altered neuro-immune interactions, enteric neuropathy and GI motility disorders. Members of the genus *Klebsiella* are known facultative anaerobe pathogenic bacteria (Li et al. 2017), which have not been implicated in T1D before, but can be a major causative agent in type 2 diabetes associated pyrogenic liver abscess (Lee et al. 2017). The lumen of the rat GI tract is an anaerobic environment (Li et al. 2017) where the facultative anaerobe *Klebsiella* may not be unprecedented but indicated an aerobic intrusion into the T1D microbiota.

While the LAM can only indirectly interact, MAM interacts both directly and indirectly with the host epithelium (Bajaj et al. 2012; Gevers et al. 2014; Van den Abbeele et al. 2011). It has been reported recently that the MAM but not the LAM of patients changed in various pathological states, like IBD or hepatic encephalopathy. These findings substantiate that mucosal host-microbiota interactions may be of importance (Sommer and Backhed 2016). Therefore, in this study our first goal was to explore the gut region-specific differences in the composition of the MAM, and secondly to determine the effect of chronic hyperglycaemia and immediate insulin treatment on the composition of MAM.

The results showed significant intestinal region-dependent differences in the composition of the MAM microbiota in the control rats. In the duodenum, the abundance of the phylum Firmicutes predominated, followed by the phylum Proteobacteria. In the ileum, besides these two phyla, Bacteroidetes also emerged, and in the colon samples, the abundance of this latter phylum further increased. Although the relative abundance of Firmicutes decreased along the GI tract, at the level of lower taxonomic units, characteristic signs of increasing microbial diversity and rearrangement of the microbiota were evident.

Our previous results showed that in STZ-induced diabetes the duodenum was the only gut segment in which a decrease in the number of nitrergic myenteric neurons was not accompanied by a decrease in the total number of neurons (Izbeki et al. 2008), and the limited diabetes-related structural alterations in the mesenteric capillaries were completely prevented by insulin treatment (Bodi et al. 2012). Interestingly, the effect of hyperglycaemia on the composition of MAM was the most prominent in the duodenum, where a massive invasion of

the phylum Tenericutes (including class Mollicutes, genus *Mycoplasma*) was observed. The presence of the aerobic *Mycoplasma* in the diabetic duodenum is unexpected. The oxygen availability has been thoroughly mapped along the GI tract of murine animals and humans (Albenberg et al. 2014; He et al. 1999; Zheng et al. 2015; Zweier et al. 2003). The various methods employed in these studies gave somewhat fluctuating oxygen levels due to both longitudinal and radial oxygen gradients (Zheng et al. 2015). *Mycoplasma* was not detected in the duodenal lumen of the T1D rats (Wirth et al. 2014). *Mycoplasma* can adhere to and to fuse with epithelial and immune cells (Rajilic-Stojanovic and de Vos 2014). In addition, the steep oxygen gradient from the anaerobic lumen towards the richly vascularized mucosa may explain the diabetes associated *Mycoplasma* attack. Although *Mycoplasma* infection has been reported in rare enteropathy (Roca-Lema et al. 2019), in IBD (Chen et al. 2001) and Crohn's disease (Roediger and Macfarlane 2002; Roediger 2004), it has not been implicated with diabetes related inflammations before (Sicard et al. 2017). A recent paper showed higher abundance of the phylum Tenericutes (class Mollicutes) in obese rats as compared to their lean counterparts. Some representatives of Mollicutes have been shown to import certain types of carbohydrates common in westernized diet for both mice and humans (e.g., glucose, fructose, and sucrose) and to metabolize these imported sugars to short-chain fatty acids, which could be readily absorbed by the host (Yan et al. 2016). Studies proved that the intestinal dendritic cells and macrophages are hyporesponsive to pathogen-associated molecular patterns (de Oliveira et al. 2017), but when epithelial barrier breakdown occurs, like in diabetes (Li and Atkinson 2015; Vaarala et al. 2008), the pattern recognition receptors, which are present in innate immune cells, recognize gut microbiota and trigger an inflammatory cascade, proinflammatory cytokine secretion, and the activation of adaptive immune responses. The number of intestinal immune cells is the lowest in the duodenum (Mowat and Agace 2014), which may augment the inflammation and prevent myenteric neuronal loss in this intestinal segment even in the presence of the pronounced reorganization of the mucosa-associated microbiota. It is perhaps of great importance that insulin treatment eliminated the genus *Mycoplasma* from the diabetic duodenum. This indicates that among other therapeutic functions of insulin, it helps to restore the healthy anaerobic microbial community in the GI tract.

In the ileum, where a significant decrease in the total number of myenteric neurons is accompanied by severe structural damage of the mesenteric capillaries in rats with STZ-induced diabetes (Bodi et al. 2012; Izbeki et al. 2008), the abundance of the phylum Proteobacteria decreased moderately, but its composition changed strikingly (Wirth et al. 2014). The class Epsilonproteobacteria, including the microaerophilic genera *Helicobacter* and *Wolinella* was basically eradicated from the diabetic samples, whereas in the controls this class represented 10% of the Bacteria.

We suppose that the high number and the diversity of immune cells in the ileum contribute to the chronic hyperglycaemia-related alterations in this gut segment. The resident microbiota regulates the development of specific subsets of lymphocytes in the gut. T helper type 17 (Th17) lymphocytes are essential in defence against bacterial infections and play roles in autoimmune disease development by producing pro-inflammatory cytokines. It was observed that the so called segmented filamentous bacteria (genetic relatives of the genus *Clostridium*), promote the generation of Th17 cells and the induction of regulatory T cells in the gut (de Oliveira et al. 2017).

Significant myenteric neuronal loss was observed earlier in the diabetic colon, which was completely prevented by insulin treatment (Izbeki et al. 2008). Unlike the ileum, the structural alterations of the microvessels remained unchanged in insulin-treated rats relative to their diabetic counterparts (Bodi et al. 2012). In the control samples the phyla Firmicutes, Bacteroidetes and Proteobacteria predominated. In the diabetic colon, the abundances of the phyla Firmicutes and Proteobacteria decreased, as described in other studies (Emani et al. 2015), whereas that of the phylum Bacteroidetes increased relative to the control rats.

The important role of gut microbiota dysbiosis was demonstrated in driving enteric neurodegeneration in mice colon via Toll-like receptor 4 exhibiting increased plasma lipopolysaccharide concentrations (Anitha et al. 2016; Reichardt et al. 2017).

Similarly to previous data (Galley et al. 2014; Van den Abbeele et al. 2011), our results show that the MAM differed substantially from LAM (Wirth et al. 2014). The duodenum presented the most prominent difference between the mucosa-associated and luminal microbiota. Although the abundance of the phylum Firmicutes was similar in the MAM and LAM, the proportions of Lactobacillales (LAM 31% vs. MAM 2%), Actinobacteria (LAM 21% vs. MAM 4 %) and Clostridia (LAM 20% vs. MAM 67%) were different.

In the ileum the differences were also significant (Lactobacillales LAM: 55% vs. MAM 0%, Clostridia LAM 1% vs. MAM 64%, Bacteroidetes LAM 0% vs. MAM 6%, Proteobacteria LAM 33% vs. MAM 23%), whereas the slightest difference was observed in the colon (Lactobacillales LAM 5% vs. MAM 1%, Clostridia LAM 33% vs. MAM 37%, Bacteroidetes LAM 40% vs. MAM 35%).

The effect of immediate insulin treatment on the

composition of MAM had a significant beneficial effect in all investigated gut segments, although, like the LAM, the normal gut flora was not totally restored.

Conclusions

In comparison to our earlier results on the LAM (Wirth et al. 2014) with the new data reported here on the MAM along the GI tract in the same STZ-induced rat model of T1D, we conclude that chronic hyperglycaemia has the most prominent effect on the LAM in the ileum, whereas in the case of MAM major changes were encountered in the duodenum. The facultative anaerobe *Klebsiella* invasion of the ileum in LAM (Wirth et al. 2014) and a similar assault by the aerobic *Mycoplasma* in the MAM indicate that local perturbation of the anaerobic environment and microbiota may play important role in the autoimmune inflammation related to T1D. This environmental factor has not been considered before and should be considered in understanding the ethology and treatment of T1D as well as similar gastrointestinal inflammatory diseases. The results presented here also confirmed two previous assumptions. First, the composition of the intestinal microbiota did not change significantly in diabetes in the colon, where the diversity of bacteria is the highest in the entire GI tract, but it changed seriously in the small intestine, which has received less attention so far in spite of the obvious diagnostic and therapeutic consequences. The variations due to the disturbed physiological status are more pronounced in the small intestine due to the lower diversity of the community. The hyperglycaemia-related alterations of microbiota were region-specific in the small intestine and were distinctly affected by insulin replacement. Second, the investigation of both the LAM and the MAM is necessary for exploring the details of the alteration of the gut microbiota in different pathological states, such as T1D. A particular attention should be paid to the oxygen gradients developing in the diseased GI tracts.

With the detailed characterization of both the LAM and MAM in the various parts of the GI tract, the intriguing question remains: how does the microbiota contribute to malnutrition in the duodenum, intestinal inflammation in the ileum and colon, and cancer in the colon? Future studies must reveal the details of the sensitive crosstalk and delicate interactions between the members of the complex microbial community and the host's epithelial barrier and immune system to combat diseases such as T1D.

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References

Albenberg L, Esipova TV, Judge CP, Bittinger K, Chen J, Laughlin A, Grunberg S, Baldassano RN, Lewis JD, Li H, Thom SR, Bushman FD, Vinogradov SA, Wu GD (2014) Correlation between intraluminal oxygen gradient and radial partitioning of intestinal microbiota. Gastroenterology 147:1055-1063 e8.

Anitha M, Reichardt F, Tabatabavakili S, Nezami BG, Chassaing B, Mwangi S, Vijay-Kumar M, Gewirtz A, Srinivasan S (2016) Intestinal dysbiosis contributes to the delayed gastrointestinal transit in high-fat diet fed mice. Cell Mol Gastroenterol Hepatol 2:328-339.

Bajaj JS, Hylemon PB, Ridlon JM, Heuman DM, Daita K, White MB, Monteith P, Noble NA, Sikaroodi M, Gillevet PM (2012) Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. Am J Physiol Gastrointest Liver Physiol 303:G675-685.

Bashir M, Prietl B, Tauschmann M, Mautner SI, Kump PK, Treiber G, Wurm P, Gorkiewicz G, Hogenauer C, Pieber TR (2016) Effects of high doses of vitamin D3 on mucosa-associated gut microbiome vary between regions of the human gastrointestinal tract. Eur J Nutr 55:1479-1489.

Bodi N, Talapka P, Poles MZ, Hermesz E, Jancso Z, Katarova Z, Izbeki F, Wittmann T, Fekete E, Bagyanszki M (2012) Gut region-specific diabetic damage to the capillary endothelium adjacent to the myenteric plexus. Microcirculation 19:316-326.

Chen W, Li D, Paulus B, Wilson I, Chadwick VS (2001) High prevalence of Mycoplasma pneumoniae in intestinal mucosal biopsies from patients with inflammatory bowel disease and controls. Dig Dis Sci 46:2529-2535.

Chermesh I, Shamir R (2009) The role of microbiota in inflammatory bowel disease. Annales Nestlé 67:27-38.

de Oliveira GLV, Leite AZ, Higuchi BS, Gonzaga MI, Mari-

ano VS (2017) Intestinal dysbiosis and probiotic applications in autoimmune diseases. Immunology 152:1-12.

Emani R, Alam C, Pekkala S, Zafar S, Emani MR, Hanninen A (2015) Peritoneal cavity is a route for gut-derived microbial signals to promote autoimmunity in non-obese diabetic mice. Scand J Immunol 81:102-109.

Flemer B, Gaci N, Borrel G, Sanderson IR, Chaudhary PP, Tottey W, O'Toole PW, Brugere JF (2017) Fecal microbiota variation across the lifespan of the healthy laboratory rat. Gut Microbes 8:428-439.

Galley JD, Yu Z, Kumar P, Dowd SE, Lyte M, Bailey MT (2014) The structures of the colonic mucosa-associated and luminal microbial communities are distinct and differentially affected by a prolonged murine stressor. Gut Microbes 5:748-760.

Gevers D, Kugathasan S, Denson LA, Vazquez-Baeza Y, Van Treuren W, Ren B, Schwager E, Knights D, Song SJ, Yassour M, Morgan XC, Kostic AD, Luo C, Gonzalez A, McDonald D, Haberman Y, Walters T, Baker S, Rosh J, Stephens M, Heyman M, Markowitz J, Baldassano R, Griffiths A, Sylvester F, Mack D, Kim S, Crandall W, Hyams J, Huttenhower C, Knight R, Xavier RJ (2014) The treatment-naive microbiome in new-onset Crohn's disease. Cell Host Microbe 15:382-392.

He G, Shankar RA, Chzhan M, Samouilov A, Kuppusamy P, Zweier JL (1999) Noninvasive measurement of anatomic structure and intraluminal oxygenation in the gastrointestinal tract of living mice with spatial and spectral EPR imaging. Proc Natl Acad Sci U S A 96:4586-4591.

Hu Y, Wong FS, Wen L (2017) Antibiotics, gut microbiota, environment in early life and type 1 diabetes. Pharmacol Res 119:219-226.

Izbeki F, Wittman T, Rosztoczy A, Linke N, Bodi N, Fekete E, Bagyanszki M (2008) Immediate insulin treatment prevents gut motility alterations and loss of nitrergic neurons in the ileum and colon of rats with streptozotocin-induced diabetes. Diabetes Res Clin Pract 80:192-198.

Jerram ST, Leslie RD (2017) The genetic architecture of type 1 diabetes. Genes (Basel) 8(8):209.

Kelly J, Daly K, Moran AW, Ryan S, Bravo D, Shirazi-Beechey SP (2017) Composition and diversity of mucosa-associated microbiota along the entire length of the pig gastrointestinal tract; dietary influences. Environ Microbiol 19:1425-1438.

Knip M, Honkanen J (2017) Modulation of type 1 diabetes risk by the intestinal microbiome. Curr Diab Rep 17:105.

Kugelberg E (2017) Microbiota: Diet can protect against type 1 diabetes. Nat Rev Immunol 17:279.

Lee IR, Sng E, Lee KO, Molton JS, Chan M, Kalimuddin S, Izharuddin E, Lye DC, Archuleta S, Gan YH (2017) Comparison of diabetic and non-diabetic human leukocytic responses to different capsule types of *Klebsiella pneumoniae* responsible for causing pyogenic liver abscess. Front Cell Infect Microbiol 7:401.

Li D, Chen H, Mao B, Yang Q, Zhao J, Gu Z, Zhang H, Chen YQ, Chen W (2017) Microbial biogeography and core microbiota of the rat digestive tract. Sci Rep 8:45840.

Li X, Atkinson MA (2015) The role for gut permeability in the pathogenesis of type 1 diabetes - a solid or leaky concept? Pediatr Diabetes 16:485-492.

Marino E, Richards JL, McLeod KH, Stanley D, Yap YA, Knight J, McKenzie C, Kranich J, Oliveira AC, Rossello FJ, Krishnamurthy B, Nefzger CM, Macia L, Thorburn A, Baxter AG, Morahan G, Wong LH, Polo JM, Moore RJ, Lockett TJ, Clarke JM, Topping DL, Harrison LC, Mackay CR (2017) Gut microbial metabolites limit the frequency of autoimmune T cells and protect against type 1 diabetes. Nat Immunol 18:552-562.

Matijašić M, Meštrović T, Paljetak HC, Perić M,Barešić A, Verbanac D (2020) Gut Microbiota beyond Bacteria—Mycobiome, Virome, Archaeome, and Eukaryotic Parasites in IBD. Int J Mol Sci 21:2668.

Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, Wilkening J, Edwards RA (2008) The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 9:386.

Mishra SP, Wang S, Nagpal R, Miller B, Singh R, Taraphder S, Yadav H (2019) Probiotics and prebiotics for the amelioration of type 1 diabetes: Present and future perspectives. Microorganisms 7(3):67.

Mowat AM, Agace WW (2014) Regional specialization within the intestinal immune system. Nat Rev Immunol 14:667-685.

Paun A, Yau C, Danska JS (2017) The influence of the microbiome on type 1 diabetes. J Immunol 198:590-595.

Qi CJ, Zhang Q, Yu M, Xu JP, Zheng J, Wang T, Xiao XH (2016) Imbalance of fecal microbiota at newly diagnosed type 1 diabetes in Chinese children. Chin Med J (Engl) 129:1298-1304.

Rajilic-Stojanovic M, de Vos WM (2014) The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol Rev 38:996-1047.

Randle-Boggis RJ, Helgason T, Sapp M, Ashton PD (2016) Evaluating techniques for metagenome annotation using simulated sequence data. FEMS Microbiol Ecol 92: fiw095.

Reichardt F, Chassaing B, Nezami BG, Li G, Tabatabavakili S, Mwangi S, Uppal K, Liang B, Vijay-Kumar M, Jones D, Gewirtz AT, Srinivasan S (2017) Western diet induces colonic nitrergic myenteric neuropathy and dysmotility in mice via saturated fatty acid- and lipopolysaccharide-induced TLR4 signalling. J Physiol 595:1831-1846.

Roca-Lema D, Martinez-Iglesias O, Fernandez de Ana Portela C, Rodriguez-Blanco A, Valladares-Ayerbes M,

Diaz-Diaz A, Casas-Pais A, Prego C, Figueroa A (2019) In vitro anti-proliferative and anti-invasive effect of polysaccharide-rich extracts from *Trametes versicolor* and *Grifola frondosa* in Colon Cancer Cells. Int J Med Sci 16:231-240.

Roediger WE (2004) Intestinal mycoplasma in Crohn's disease. Novartis Found Symp 263:85-93; discussion 93-98, 211-218.

Roediger WE, Macfarlane GT (2002) A role for intestinal mycoplasmas in the aetiology of Crohn's disease? J Appl Microbiol 92:377-381.

Sicard JF, Le Bihan G, Vogeleer P, Jacques M, Harel J (2017) Interactions of intestinal bacteria with components of the intestinal mucus. Front Cell Infect Microbiol 7:387.

Sommer F, Backhed F (2016) Know your neighbor: Microbiota and host epithelial cells interact locally to control intestinal function and physiology. Bioessays 38:455-464.

Tanca A, Palomba A, Fraumene C, Manghina V, Silverman M, Uzzau S (2018) Clostridial butyrate biosynthesis enzymes are significantly depleted in the gut microbiota of nonobese diabetic mice. 3(5):e00492-18.

Tian J, Li M, Lian F, Tong X (2017) The hundred most-cited publications in microbiota of diabetes research: A bibliometric analysis. Medicine (Baltimore) 96:e7338.

Vaarala O, Atkinson MA, Neu J (2008) The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. Diabetes 57:2555-2562.

Van den Abbeele P, Van de Wiele T, Verstraete W, Possemiers S (2011) The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS Microbiol Rev 35:681-704.

Wang Y, Devkota S, Musch MW, Jabri B, Nagler C, Antonopoulos DA, Chervonsky A, Chang EB (2010) Regional mucosa-associated microbiota determine physiological expression of TLR2 and TLR4 in murine colon. PLoS One 5:e13607.

Wirth R, Bodi N, Maroti G, Bagyanszki M, Talapka P, Fekete E, Bagi Z, Kovacs KL (2014) Regionally distinct alterations in the composition of the gut microbiota in rats with streptozotocin-induced diabetes. PLoS One 9:e110440.

Yan X, Feng B, Li P, Tang Z, Wang L (2016) Microflora disturbance during progression of glucose intolerance and effect of sitagliptin: An animal study. J Diabetes Res 2016:2093171.

Zheng L, Kelly CJ, Colgan SP (2015) Physiologic hypoxia and oxygen homeostasis in the healthy intestine. A review in the theme: cellular responses to hypoxia. Am J Physiol Cell Physiol 309:C350-360.

Zweier JL, He G, Samouilov A, Kuppusamy P (2003) EPR spectroscopy and imaging of oxygen: applications to the gastrointestinal tract. Adv Exp Med Biol 530:123-31.