Butyrate modulates diabetes-linked gut dysbiosis: epigenetic and mechanistic modifications

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Abstract

Diabetic dysbiosis has been described as a novel key player in diabetes and diabetic complications. However, the cellular/molecular alterations associated with dysbiosis remain poorly characterized. For that, control, non-obese type 2 diabetic MKR mice and MKR mice treated with butyrate were used to delineate the epigenetic, cellular and molecular mechanisms by which dysbiosis associated with diabetes induces colon shortening and inflammation attesting to gastrointestinal disturbance. Our results show that dysbiosis is associated with T2DM and characterized by reduced Bacteroid fragilis population and butyrate-forming bacteria. The reduction of butyrate-forming bacteria and inadequate butyrate secretion result in alleviating HDAC3 inhibition and altering colon permeability. The observed changes are also associated with an increase in ROS production, a rise in NOX4 proteins, and a shift in the inflammatory markers, where IL-1β is increased and IL-10 and IL-17α are reduced. Treatment with butyrate restores the homeostatic levels of NOX4 and IL-1β. In summary, our data suggest that in T2DM, dysbiosis is associated with a reduction in butyrate content leading to increased HDAC3 activity. Butyrate treatment restores the homeostatic levels of the inflammatory markers and reduces ROS production known to mediate diabetes-induced colon disturbance. Taken together, our results suggest that butyrate could be a potential treatment to attenuate diabetic complications.

Introduction

Type 2 diabetes mellitus (T2DM) has a wide range of etiologies, intricate pathogenesis and predisposes for various serious micro- and macro-vascular complications. Diabetes mellitus is strongly associated with disturbance of gut healthy microbiota (Mejía-León & Barca 2015, Haro et al. 2016), a condition known as diabetic dysbiosis. Evidence from literature suggests that gut microbiota plays a central role in the pathogenesis of T2DM (Manco et al. 2010, Musso et al. 2010, Esteve et al. 2011); yet, these studies relied on statistical correlations and educated hypotheses with no mechanistic foundations. Therefore, studies are warranted to delineate the molecular mechanisms by which gut microbiota mediates the pathogenesis of T2DM and its complications.

Several research groups tried to establish a microbial signature for type 1 and type 2 DM; however, to our

Despite the complex community of microorganisms that inhabit the digestive tract, and regardless of the conflicting findings, all of these studies were able to identify a different flora signature for T1DM, T2DM and obesity (Mejía-León & Barca 2015, Navab-Moghadam et al. 2017). However, the published findings further confirm the role for microbiota in the pathogenesis of metabolic alterations.

By to by, mechanistically, gut microbiota affects the intestinal immune system directly and impacts the lymphocyte-homing receptors which can lead to the development of autoimmune diabetes (Furusawa et al. 2013, Khan & Jena 2014, 2015, Meier & Wagner 2014, Dirice et al. 2017), which can explain the alteration in the inflammatory markers observed in diabetes. Furthermore, another mechanistic factor that has been shown to play a role in inflammation is epigenetic modification through histone deacetylation.

In that spirit, butyrate is a widely known histone deacetylation (HDAC) enzyme inhibitor (Gao et al. 2009, Donohoe et al. 2012). HDAC inhibition by butyrate regulates cellular proliferation, differentiation and energy metabolism and has been described to play a direct role in the pathogenesis of DM (Li et al. 2010, Canani et al. 2011) by increasing the differentiation and the gene expression of insulin in β-cells (Goicoa et al. 2006). Moreover, butyrate was associated with reduction of plasma glucose, insulin resistance and body weight in diabetic mice fed with high-fat diet (Anderson et al. 2009, Canani et al. 2011). Moreover, butyrate deficiency was portrayed to alter glucose metabolism and to aggravate diabetic pathogenesis (Khan & Jena 2015) by inducing inflammation and oxidative stress (Furusawa et al. 2013, Khan & Jena 2014).

From the HDAC family of enzymes that epigenetically regulate cellular processes, HDAC3 appears to be the most significant in mediating diabetic pathogenesis. HDAC3-selective inhibition can actually limit pancreatic islet infiltration, abolish the death of β-cells and protect against development of T1DM in non-obese diabetic mice (Dirice et al. 2017). In their review on the effect of HDAC3 inhibition, Meier and Wagner concluded that selective inhibition of HDAC3 could be a promising strategy in the treatment of metabolic diseases (Meier & Wagner 2014). Furthermore, HDAC3 was associated with the onset and development of diabetic complications.

A clinical association study assessed the activity of HDAC3 in peripheral blood mononuclear cells of patients with T2DM and found that its activity was higher than that of the control. Yet, the underlying cause of the observed effect was not well highlighted despite the subtle hint of an alteration in the levels of the systemic inflammatory cytokines (Sathishkumar et al. 2016). When it comes to epigenetic alteration, HDAC3 inhibition prevented diabetic cardiomyopathy onset by regulating DUSP5-ERK1/2 pathway in OVE26 mice (Xu et al. 2017). Likewise, inhibition of HDAC3 protected diabetic mice from diabetes-induced liver damage (Zhang et al. 2018).

Another key inducer of diabetic complications is the increase in reactive oxygen species (ROS) production. Our group and others show that NADPH oxidase-4 (NOX4) is an important mediator for diabetic nephropathy, neuropathy, retinopathy and cardiomyopathy (Al-Shabrawey et al. 2008, Gray et al. 2013, Zhao et al. 2014, Thallas-Bonke et al. 2015, Eid et al. 2016, Prakoso et al. 2017). Several studies showed that the expression of NOXs is under the regulation of HDAC3 (Siuda et al. 2012). However, the crosstalk between NOXs and HDAC3 is not described in a diabetic context. This allured us to investigate if NOXs expression is correlated with HDAC3 inhibition in diabetes-induced gastrointestinal (GI) complications.

In this study, we show that non-obese type 2 diabetic mice develop dysbiosis characterized by reduction of butyrate-forming bacteria and inadequate butyrate secretion. These in turn result in alleviating HDAC3 inhibition. The activation of HDAC observed in the non-obese type 2 diabetic mice aggravates inflammation, increases NOXs-induced ROS production and alters colon permeability. Butyrate treatment reversed the observed physio-pathological changes and restored colon anatomy. Aberrant alteration of the inflammatory markers and the increase in ROS production through NOX4 activation could potentially explain the development of diabetes-induced GI complications observed in the diabetic mice reflected by colon shortening and inflammation.

**Materials and methods**

**Animal studies**

All animal procedures described in the manuscript were done in accordance with the United States Public Health Service’s Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the American University.
Of Beirut. All mice were housed in the animal care facility, American University of Beirut, and were fed with autoclaved food ad libitum and maintained in fixed temperature and humidity in 12-h light/darkness cycles (light from 5:00 h to 17:00 h and dark from 17:00 h to 5:00 h). Our present study included MKR (muscle IGF-I receptor (IGF-IR)-lysine-arginine) male mice that were purchased from The Jackson Laboratory. MKR male mice have a mutation in their skeletal muscle insulin receptor (dominant-negative mutant IGF-IRs) imparting insulin resistance in muscle, liver, and adipose tissue, leading to the development of T2DM at adulthood (Fernández et al. 2001, Kim et al. 2006). The MKR mice were developed on FVB-NJ background, so these WT mice were used as controls. These mice have been shown to develop diabetic GI complications resembling those seen in humans. In this study, the mice were divided as follows: group (1) FVB-NJ, the non-obese control mice; group (2) non-obese diabetic MKR mice treated with vehicle (PBS) and group (3) non-obese diabetic MKR mice treated with 20 mg/kg of sodium butyrate (Sigma-Aldrich) dissolved in PBS. All injections were administered daily via intraperitoneal route (IP) and started at 23 weeks of age and continued for 8 weeks. The dose of 20 mg/kg of butyrate is sufficient to provide 10 µM of butyrate concentration in the plasma which is reported to be the concentration of a healthy gut milieu (Nishitsuji et al. 2017). At the end of the experiments (31 weeks of age), fecal samples were collected from each mouse under sterile conditions and stored at −80°C in a fridge for further extraction of bacterial genomic DNA for microbial identification.

**Organ harvesting**

At 31 weeks of age, all mice were killed by exsanguination of blood from the heart after anaesthetizing them with isoflurane. Subsequently, distal colon segments were harvested for protein and mRNA extraction and determination of HDAC3 and HAT activity in colon tissues. Colon lengths, from just underneath the cecum till the anus, were recorded to assess the effect of diabetes and butyrate treatment on the colon anatomy. Blood was collected via cardiac puncture under anaesthesia in heparin vacutainers for determination of inflammatory cytokines. In total, 1 mL of blood was withdrawn from the heart. Blood was centrifuged at 1500 g for 15 min at 4°C and plasma was collected and stored at −80°C for further analysis. Cecal and fecal contents were collected for the determination of their butyrate content.

**Determination of random blood glucose and circulatory cytokines**

Random blood glucose, obtained from the tail vein (5 µL), was measured weekly between 10:00 h and noontime using a glucometer (Accu-Chek Performa, Roche) utilizing the glucose oxidase-peroxidase method. Random blood glucose measurements were started at 7 weeks of age and were maintained throughout the whole period of the study. During killing, plasma inflammatory markers were determined using Inflammatory Cytokines Multi-Analyte ELISA Kits (Cat. No. MEM-004A, Qiagen). This multi-array kit detects mouse inflammatory cytokines using a conventional ELISA protocol. This kit was used to determine circulatory interleukin IL-10, IL-17α, tumor necrosis factor (TNF)-α and granulocyte-colony-stimulating factor (G-CSF) according to the manufacturer’s instructions.

**Determination of glycated hemoglobin (HbA1c)**

An aliquot of the whole blood (50 µL) was used for the determination of HbA1c to determine the presence of chronic diabetic state. HbA1c levels were determined with a Mouse HbA1c Kit (Catalogue #80310, Crystal Chem, USA), according to the manufacturer’s instructions and normalized against total haemoglobin to get HbA1c %.

**Isolation of fecal DNA and microbial gene analysis**

Feces were collected at different time points from all mice under aseptic conditions and stored at −80°C for further analysis. Bacterial DNA was extracted from feces using DNeasy PowerSoil Kit (Cat. No. 12888-100, Qiagen) according to the manufacturer’s instructions. Real-time PCR (RT-PCR) was used to determine the different microbial communities. Herein, we studied the butyrate-forming bacterial communities that were previously described by Vital et al. as the major contributors of butyrate formation (Vital et al. 2017). Bacterial communities were identified using specific primers for these communities and for the butyrate kinase gene (Table 1). Results were normalized against 16S rRNA gene levels in each sample and were compared to controls.

**Real-time PCR**

Real-time PCR was conducted using Bio-Rad CFX384 RT-PCR system using 10 ng of DNA, 300 nM of each
Table 1  List of primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reverse</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Akkermansia muciniphila</td>
<td>CAGCACGTGAAGTGCGTTGGGAC</td>
<td>CTTTGCGGTGGCTTGACGATT</td>
<td>Schneeberger et al. 2015</td>
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<td>Aklistipes spp.</td>
<td>TTAGAGATGGGCACTGGTTG</td>
<td>TGAATCCTCCCGATTT</td>
<td>Roager et al. 2014</td>
</tr>
<tr>
<td>Anaerostipes spp.</td>
<td>GCCTAGTGGCATGTAAGT</td>
<td>CTGCACTCCAGCATGACG</td>
<td>Chia et al. 2018</td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>GAAGGTCCCTCCACAATTG</td>
<td>CGCACCCTGGCCTGTTCA</td>
<td>Kennedy et al. 2014</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>ATACCGAGAGCTGCGACCCTGAAT</td>
<td>CTGGTCACTCCCGATGCA</td>
<td>Sánchez et al. 2012</td>
</tr>
<tr>
<td>Butyrate kinase</td>
<td>CAGGCCTAACAATCAGAAGTC</td>
<td>GATACACGCTGGCTTCATTGA</td>
<td>Yadav et al. 2013</td>
</tr>
<tr>
<td>Butyricoccus spp.</td>
<td>ACCCTGAGAATAGGCTC</td>
<td>GTCAGTAGCAGTCCAG</td>
<td>Eeckhaut et al. 2013</td>
</tr>
<tr>
<td>Butyrivibrio spp.</td>
<td>CTATCAAGAGGGGAAAGAAAG</td>
<td>GUGGCAGAGGCAAGAAG</td>
<td>Kennedy et al. 2014</td>
</tr>
<tr>
<td>Coprooccus comes</td>
<td>GTGACCCGGGTTGAAGCTGGCA</td>
<td>CATCGCTGCCACTTCTGAC</td>
<td>Ramirez-Farias et al. 2008</td>
</tr>
<tr>
<td>Coprooccus eutactus</td>
<td>CTGGAGCTTCTGCTCGAGCTCATATT</td>
<td>GTTCGCGCTGCTGTAC</td>
<td>Kurakawa et al. 2015</td>
</tr>
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<td>Enterobacteribacter</td>
<td>GACGCGCAGGAGCA</td>
<td>CACGTACACCCCAACG</td>
<td>Kurakawa et al. 2015</td>
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<tr>
<td>Eubacterium hallii</td>
<td>GTGTCGCCGCACTTCTGAC</td>
<td>GTTGCACTCCCGATGCA</td>
<td>Kageyama &amp; Benno 2001</td>
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<td>Eubacterium rectale</td>
<td>CATTGCTCCGCTGCGCTGTC</td>
<td>ATGTGACCGGCTGCCAGC</td>
<td>Kurakawa et al. 2015</td>
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<td>Eubacterium ventriosum</td>
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<td>Faecalibacterium prausnitzii</td>
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<td>GGCGCTCTTTTACACCCA</td>
<td>Kennedy et al. 2014</td>
</tr>
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<td>Lachnospiraceae</td>
<td>CGGATAGTTATGCAAGAAGC</td>
<td>AGTCTTCGCTCCCAAG</td>
<td>Tong et al. 2011</td>
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<td>Odoribacter splanchinicus</td>
<td>ATGTTGAAGAGGCAAAGG</td>
<td>GGTCCTGACCTCCCAAG</td>
<td>Walker et al. 2011</td>
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<tr>
<td>Oscillospira spp.</td>
<td>AGGCAGTACCCCTGGAATAGGC</td>
<td>TCCCACCAAGCACTTATGTG</td>
<td>Kurakawa et al. 2015</td>
</tr>
<tr>
<td>Roseburia intestinalis</td>
<td>GCACAGGCAGTGATGCTGCTGAC</td>
<td>AACACATCACTTCTGTCATC</td>
<td>Kennedy et al. 2014</td>
</tr>
<tr>
<td>Ruminococcusaceae</td>
<td>TAAAACACAATTAGATWATCCACCTGG</td>
<td>ACCTTCCGCGTGTGGCAAC</td>
<td>Kennedy et al. 2014</td>
</tr>
<tr>
<td>Universal 16S rRNA</td>
<td>GTGSTGCAAGYYGTYGTGCA</td>
<td>AGCAGCTCGCCMCNCTCTC</td>
<td>Kennedy et al. 2014</td>
</tr>
</tbody>
</table>

Determination of histone acetylase and histone deacetylase 3 activities

The enzymatic activities of HAT and HDAC3 in distal segments of colon tissues were determined using HAT and HDAC3 Activity Assay Kits (Cat. No. EPI001 & EPI004, Sigma-Aldrich) according to the manufacturer’s instructions.

Determination of fecal and cecal butyrate content

High-performance liquid chromatography (HPLC)/mass spectrometry (MS) technique was used to quantify butyrate content in feces and cecal contents of mice according to the method described by Parise et al. (2008). The LTQ-FT system (Thermo Scientific) was used with the application of a Phenomenex Synergi Polar-RP column (Phenomenex, USA). Results are expressed as mg butyrate per g of feces or cecal content.

Detection of reactive oxygen species (ROS) production in colon tissues using HPLC

Determination of ROS production in distal segments of colon tissues was assessed using HPLC method as previously described (Eid et al. 2016). Briefly, sections of the colons were washed twice with Hanks’ balanced salt solution (HBSS)-diethylenetriaminepentaacetic acid (DTPA), homogenized and incubated for 30 min with 50 μM DHE (Sigma-Aldrich) in HBSS–100 μM DTPA. This step was followed by the addition of acetonitrile and centrifugation at 12,000 g for 10 min at 4°C. The homogenate was dried under vacuum and analysed by HPLC with fluorescence detectors. Quantification of DHE, EO, and ethidium concentrations was performed by comparison of integrated peak areas between the obtained and standard curves of each product under chromatographic conditions identical to those described earlier. EOH and ethidium were monitored by fluorescence detection with excitation at 510 nm and emission at 595 nm, whereas DHE was monitored by UV absorption at 370 nm. The results are expressed as the amount of EOH produced (nmol) normalized for the amount of DHE consumed (i.e. initial minus remaining DHE in the sample; μmol).

Primary culture

To assess the role of HDAC in regulating NOX4 protein expression, part of the distal segments of colon of control
mice (FVB-NJ) was extracted and used for ex vivo culture (Udden et al. 2017). Briefly, the extracted colons were cleaned with ice-cold PBS and feces were completely removed. Afterwards, the colon was cut into pieces with 1 cm length and weighed. For the organ culture, a cell strainer (100 μm) was placed on a six-well plate to which all the colon pieces were transferred. Dulbecco’s modified eagle’s media (DMEM) was used and was supplied with 2% FBS and 1% Penicillin-Streptomycin (1×). The colon was left to rest in the media for 24 h, before incubating the pieces with either normal glucose NG (5 mM), high glucose (25 mM), high glucose with sodium butyrate (4 mM), high glucose with the HDAC-specific inhibitor (Trichostatin A ‘TSA’, 0.5 μM) or high glucose with combination of butyrate and TSA for 48 h, before proceeding to protein extraction.

Western blot

Total proteins from homogenates of the distal segments of the colon were obtained using 200 μL of radioimmune precipitation assay buffer containing 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L NaVO4, 1 mmol/L PMSF, 20 μg/mL aprotinin, 20 μg/mL leupeptin, and 1% NP-40 and then incubated overnight on a rotator at 4°C. Subsequently, samples were centrifuged at 13,700 rpm for 30 min at 4°C. Total protein content of each sample was quantified using the Lowry Protein Assay (Peterson 1977). Samples (containing 30 μg of proteins) were loaded on 15% SDS-PAGE and transferred to nitrocellulose membrane. Blots were incubated with rabbit polyclonal anti-NOX4 (1:500, Santa Cruz), rabbit polyclonal anti-IL-1β (1:500, Abcam), mouse polyclonal HSC-70 (1:1000, Santa Cruz). The primary antibodies were detected using horseradish peroxidase-conjugated IgG (1:20,000). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using National Institutes of Health Imaging software.

Statistical analysis

Statistical analysis was performed using Graphpad Prism Software (Graphpad, version 6.0) (Motulsky 1999). Sample size was calculated to give 80% power and P ≤ 0.05. All the results are expressed as mean±s.d. We used a two-tailed Student’s t test or ANOVA (one-way or two-way) to determine the significance and P ≤ 0.05 was considered statistically significant. We used Levene’s test to test for differences in group variances and chose the t test calculation method accordingly.

Results

**MKR mice develop type 2 diabetes without obesity and suffer from gastrointestinal dysfunction**

Body weight and random blood glucose levels of the MKR mice and their FVB-NJ littermates were measured weekly (from 7 to 31 weeks of age). Although the body weight of the MKR mice was statistically comparable to that of the controls (indicating no signs of developing obesity) (Fig. 1A), random blood glucose levels were significantly different between the two groups of mice (Fig. 1B). Furthermore, chronic hyperglycemia in diabetic MKR mice was assessed by HbA1c % and was significantly elevated in diabetic mice compared to their control littermates (Fig. 1C). Noteworthy, there was no significant difference in random blood glucose or HbA1c % between MKR mice treated with butyrate and that of the untreated MKR mice (Fig. 1B and C).

Moreover, we measured colon lengths of all mice groups to determine gastrointestinal dysfunction due to diabetes and its associated dysbiosis. Colons of MKR mice were significantly shorter compared to their control littermates. This was reversed upon treatment with butyrate (Fig. 1D).

**Diabetic mice microbiota contains less Bacteroid fragilis and butyrate-forming bacteria**

In order to assess the difference in gut microbiota that resulted from the disturbance in carbohydrate metabolism, RT-PCR, using specific primers for different bacterial communities and primers for the total butyrate kinase genes (Table 1), was performed. Our results show the presence of a significant difference between the microbiota of the MKR mice and that of the controls that is highlighted by the reduction of B. fragilis and the most relevant butyrate-forming bacteria (Vital et al. 2017) (Fig. 2A), Butyricoccus spp., Butyrivibrio spp., Coprococcus comes, Eubacterium hallii, Eubacterium rectale, Eubacterium ventriosum, and Roseburia intestinalis (Fig. 2B and C). These findings were also reflected by the reduced abundance of the total butyrate kinase genes (Fig. 2B). Furthermore, the MKR mice and their control littermates had similar abundance of Akkermansia muciniphila, Bacteroidaceae, Enterobacteriaceae, Faecalibacterium prausnitzii, Lachnospiraceae and Ruminococcaceae that were previously shown to be associated with other metabolic conditions (Li et al. 2011a, Everard et al. 2013, Shin et al. 2014, Kameyama & Itoh 2014, Lukovac et al. 2014, Roager et al. 2014, Roopchand et al. 2015, Schneeberger et al. 2015).
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The microbiota composition in diabetic mice has been studied by Dao et al. (2016), Lippert et al. (2017), and Plovier et al. (2017). These studies suggest that diabetes-associated dysbiosis affects mainly the butyrate-forming bacterial communities without any significant difference on the level of bacterial families.

Diabetes is coupled to a decrease in cecal and fecal butyrate content, a higher activity of the colon histone deacetylase 3 (HDAC3) and alteration of inflammatory cytokines

Since T2DM is correlated with less abundance of butyrate-forming bacterial population, we measured the cecal and fecal butyrate contents of MKR and FVB-NJ control mice. Both cecal and fecal butyrate contents were significantly lower in MKR mice (Fig. 3A), correlating with their reduced content of the butyrate-forming bacteria.

One of the main biological actions of butyrate is its role as an HDAC inhibitor. As MKR diabetic mice showed less abundance of butyrate, we assessed the activity of HDAC3 in colon tissues. As expected, MKR mice had higher HDAC3 activity compared to their control littermates (Fig. 3B). Likewise, we assessed the activity of HAT, and our results show no significant differences between the activities of HAT of MKR mice when compared to those of their control littermates suggesting that butyrate affects HDAC3 without inhibiting HAT (Fig. 3B).

It is thought that dysbiosis in DM plays a key role in the alteration of the inflammatory state (Cani et al. 2007, 2012, Everard & Cani 2013). Likewise, butyrate-forming bacteria, showed to be altered in our diabetic model, may affect the host immunity and the inflammatory state. For that, we assessed the levels of circulatory cytokines in plasma of MKR and control mice. Our results show that the levels of the anti-inflammatory cytokine IL-10 and the...
pro-inflammatory IL-17α are significantly lower in MKR mice when compared to their control littermates (Fig. 3C). Moreover, the plasma levels of IL-12, TNF-α and G-CSF did not show any significant difference between the two groups (Fig. 3C). In parallel, the protein expression of IL-1β, which is the major cytokine involved in diabetic pathogenesis and diabetic complications (Hasegawa et al. 1991, Blakemore et al. 1996, Larsen et al. 2007a, Mandrup-Poulsen et al. 2010, Banerjee & Saxena 2012), in colon tissue of MKR mice was significantly upregulated when compared to control mice. Of interest, treatment with sodium butyrate restored back the homeostatic levels of IL-1β (Fig. 3D). Taken together, these results highlight the implication of the butyrate-forming bacteria in the diabetes-induced inflammatory state modification.

Butyrate treatment reverses diabetes-induced ROS production and NADPH oxidase 4 protein expression through HDAC3 inhibition

Diabetes is described to be associated with increased NADPH oxidases-induced reactive oxygen species production (Maalouf et al. 2011, Fitzgerald et al. 2012, Eid et al. 2013a,b, 2016). As expected, ROS levels were increased in the colon of the MKR mice, and this was correlated with increased NOX4 protein expression (Fig. 4A and B). Importantly, treatment with butyrate reversed the increase in ROS production and reduced NOX4 protein expression (Fig. 4A and B). Taken together, these results suggest that diabetes-induced dysbiosis is associated with an alteration in ROS production through an upregulation of NOX4.

Previous data from the literature suggest that the expression of NOX4 is under strong epigenetic regulation by HDAC3 (Hakami et al. 2016). To investigate if the reduction of NOX4 protein expression observed with butyrate treatment was the result of HDAC3 inhibition, primary culture of colon tissues extracted from the control mice were incubated with either normal glucose NG (5 mM), high glucose (25 mM) in the presence or absence of sodium butyrate (4 mM), high glucose with the presence or absence of TSA (0.5 µM), an HDAC-specific inhibitor, or with high glucose in the presence or absence of a combination of butyrate and TSA. After 48 h of incubation, our results show that cells exposed to high glucose had an increased expression of NOX4, mimicking the observed upregulation of NOX4 in the diabetic mice. Importantly, NOX4 overexpression was reversed when the incubated cells with high glucose were treated with butyrate or TSA. Of interest, the combination
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Our data show that untreated diabetic mice. Here, we report a microbial signature unique to the hyperglycemia associated with T2DM without the obesity component and elucidate the molecular mechanisms by which microbiota, and its secreted butyrate, contribute to the diabetic pathogenesis and complications.

We used MKR mice models which are non-obese mice that develop T2DM in adulthood due to a mutation in the insulin receptor in their muscles (Fernández et al. 2001). During the whole period of the study, these mice had high levels of Hba1c and similar body weights when compared to the controls confirming the non-obese insulin-resistant diabetic phenotype. Furthermore, food consumption was monitored weekly and the mean food intake was higher in MKR mice compared to that of the control group (data not shown). The higher intake of food may explain why the diabetic MKR mice maintained their weight despite the progression of diabetes.

Besides, shortening of colons is considered a hallmark of gastrointestinal complications (Chassaing et al. 2014, Ko et al. 2014). Our data show that untreated MKR mice had shorter colons compared to the control and the butyrate-treated groups reflecting gastrointestinal complications and colitis due to diabetes. Taken together, these data suggest that butyrate protected against diabetes-associated gastrointestinal complications reflected by preservation of the normal colon length, protecting it from shortening and inflammation.

Concerning the differential microbial population signature, our results show that B. fragilis and butyrate-forming bacteria were reduced in mice with T2DM when compared to their control littermates. Both MKR mice and their control littermates had similar abundance of Akkermansia muciniphila, Bacteroidaceae, Enterobacteriaceae, Faecalibacterium prausnitzii, Lachnospiraceae and Ruminococcaceae. The reduction of B. fragilis in the microbiota of patients with T2DM was reported by Navab-Moghadam et al. (2017). Many studies focused on the reduction of F. prausnitzii and other butyrate-forming bacteria in obesity and T2DM (Lau et al. 2011, Karlsson et al. 2012), here, we report the development of its complications. Noteworthy, all the association studies between gut microbiota and DM that included obesity could not differentiate if the reported microbial signature is associated with diabetes, obesity or to both co-morbidities. Noteworthy, the bias of obesity previously reported were performed in both mice models as well as in clinical settings (Musso et al. 2010, Vijay-Kumar et al. 2010, Esteve et al. 2011, Karlsson et al. 2012, Everard & Cani 2013, Ellekilde et al. 2014, Grasset et al. 2017). Our study was performed to establish a microbial signature that contributes to its pathogenesis and the development of its complications.

Discussion

Type 2 DM is associated with disturbance of gut microbiota that contributes to its pathogenesis and the vicious cycle of development of its complications. Reactive oxygen species (ROS) production and protein expression of NADPH oxidase (NOX4) in all mice groups (n ≥ 3) and in primary culture of colon tissue. Data are expressed as mean ± s.d. (A) Scatter plot representing relative ROS production in all mice groups. (B) Representative blots and scatter plot representing the quantification of relative protein expression of NOX4 in all mice groups. (C) Representative blots and scatter plot representing the quantification of relative protein expression of NOX4 in colon tissues cultures in normal glucose and high glucose media (either treated with vehicle, butyrate, trichostatin A or a combination of butyrate and trichostatin A for 48 h). * statistically significant at P < 0.05 vs control. # statistically significant at P < 0.05 vs MKR diabetic mice. HG, high glucose; NG, normal glucose; NOX, NADPH oxidase; TSA, trichostatin A.

of TSA and butyrate did not show a different effect on NOX4 expression when compared to the monotherapy treatment. These results suggest that butyrate effect is mediated by HDAC inhibition (Fig. 4C).
similar reduction for butyrate-forming bacteria with same abundance of *F. prausnitzii*. Although an association was previously reported between an increase of Bacteroidaceae (Lau et al. 2011) and reduction of *A. muciniphila* and T2DM mellitus (Everard et al. 2013, Shin et al. 2014), our non-obese MKR mice did not show similar signature suggesting that the previous reported associations may have been due to obesity rather than T2DM.

As for the Lachnospiraceae and Ruminococcaceae families, both contain butyrate-forming species. Our results show that the abundance of these two families was comparable between MKR and control mice, while MKR mice show lower abundance of butyrate-forming bacteria. This may have been due to an increased abundance of butyrate non-forming bacteria in both families that compensated for the observed reduction of the butyrate-forming proportion.

Furthermore, performing a deeper analysis of the butyrate-forming bacterial species confirmed these results where the MKR mice had lower abundance of *Butyricicoccus spp.* (from Ruminococcaceae family) and *Butyrivibrio spp.*, *Coprococcus comtes*, *Eubacterium hallii*, *Eubacterium rectale*, *Eubacterium ventriosum*, and *Roseburia intestinali* (from the Lachnospiraceae family) compared to that of their control littermates.

The observed reduction in butyrate-forming bacteria resulted in reduced secretion of butyrate in cecal and fecal contents. The reduction of butyrate may be responsible for some of the pathological findings in T2DM due to HDAC3 activation (Furusawa et al. 2013, Khan & Jena 2014). As an anti-inflammatory, butyrate inhibits HDAC3 which restores the peroxisome proliferation-activated receptor (PPAR)-γ and liver-X receptor (LXR) function in obese rats (Li et al. 2011b). Additionally, butyrate can bind to free fatty acid receptors (FFA) 1 and 2, which modulate immune responses (Masui et al. 2013). Whether these effects are solely due to HDAC inhibition or histone acetylation or non-histone targets is still unknown (Kaji et al. 2014, Remely et al. 2014). Our diabetic MKR mice also showed enhanced HDAC3 activity compared to the controls in colon tissues. The correlation between T2DM and increased HDAC3 activity was reported previously by Sathishkumar et al. without explaining the underlying cause (Sathishkumar et al. 2016). In our study, we potentially fill the missing link by showing that the correlation between T2DM and the increase in HDAC3 activity is due to the reduction of gut butyrate production.

Another confirmation for this regulation was shown when the inhibition of HDACs by TSA, β-hydroxybutyrate or SAHA reduced the expression of NOX4 in endothelial cells, spinal cord tissues and human aortic smooth muscles cells (Hakami et al. 2016, Kong et al. 2017, Manea et al. 2018). Our results confirm this observation, where TSA attenuates diabetes-induced NOX4 protein expression. Moreover, our results are also in line with the finding of Chen et al. where chromatin immunoprecipitation (ChIP) and luciferase promoter assays showed that the expression of NOXs are under direct control of HDAC in immune cells (Chen et al. 2016a).

HDAC3 epigenetically regulates inflammation and metabolic pathways related to diabetic pathogenesis (Meier & Wagner 2014). Genome-wide ChIP studies showed that HDAC inhibition can result in anti-inflammatory responses (Rafehi et al. 2014). Many studies emphasized that inhibition of HDAC3 attenuates diet-induced metabolic dysfunction (McGee-Lawrence et al. 2015) and hyperglycemia by regulating the transcriptional activity of FoxO1 (Cho et al. 2018). In adult β-cells, deletion of HDAC3 increases the secretion of insulin and improves glucose tolerance (Remsberg et al. 2017) and prevents cytokine-induced (mainly IL-1β) apoptosis (Larsen et al. 2007b). However, data from the literature showed that depletion of HDAC3 can induce glucose intolerance and increase susceptibility for insulin resistance in islet beta cells (Chen et al. 2016b, Hong et al. 2017). These findings suggest that although partial inhibition of HDAC3 is beneficial in T2DM, its total depletion can impact the glucose metabolism profoundly. The work on HDAC3 inhibitors has been culminated with the virtual screening and *in vitro* testing of novel inhibitors that can be used as anti-diabetic drugs (Xia et al. 2018). Moreover, a selective HDAC3 inhibitor, LW3, has been recently discovered confirming the druggability of this important epigenetic regulator (Grydet et al. 2019).

In our study, we show that the protein expression of IL-1β significantly increased as a result of T2DM. Increased IL-1β has been previously correlated with specific commensal bacterial abundance such as Streptococcus, Prevotella, Haemophilus and Veillonella spp. (Said et al. 2013) in inflammatory bowel syndrome. High plasma levels of IL-1 are associated with many auto-immune diseases including T1DM and blockage of IL-1 pathway was shown to be beneficial both in T1DM and T2DM (Larsen et al. 2007a, Mandrup-Poulsen et al. 2010).

Beside, butyrate and PSA (produced by *B. fragilis*) are important for the maturation of T-cells that produce anti-inflammatory IL-10 and pro-inflammatory IL-17 (Bereswill et al. 2011, Park et al. 2015). Since we have observed a reduction of *B. fragilis* and butyrate-forming bacterial population together with reduced production of cecal and...
fecal butyrate, we found that circulatory levels of IL-10 and IL-17 are decreased in comparison to the controls. IL-10 is protective against progression of DM as Robert et al. reported that delivery of IL-10 can actually reverse DM in non-obese diabetic (NOD) mice (Robert et al. 2014). The role of IL-17 in DM is paradoxical. Although it is a pro-inflammatory marker, its reduced plasma levels were associated with diabetic nephropathy and retinopathy and were negatively correlated with BMI, T2DM duration and glycated haemoglobin (Chen et al. 2016c, Galvan & Danesh 2016).

In parallel, we investigated the protein expression of NOX4 that is involved in key pathways of diabetic complications (Maalouf et al. 2011, Fitzgerald et al. 2012, Eid et al. 2013a,b, 2016). We found that their protein expressions were higher in MKR diabetic mice when compared to the controls. This is concomitant with other studies on DM (Siuda et al. 2012, Gray et al. 2013, Thallas-Bonke et al. 2015, Eid et al. 2016). This is the first study to molecularly correlate the elevated expression of NOXs to increased activity of HDAC3 due to the reduction of butyrate production.

Taken together, our findings underline the beneficial effects of butyrate supplements on the gut health without affecting the gut microbiota composition. These results corroborate with other studies that presented that butyrate, through acting as an HDAC inhibitor and anti-inflammatory agent, could impart protection against colon inflammation and gastrointestinal complications (Davie 2003, Scharlau et al. 2009, Vieira et al. 2012, Ye & Gao 2013). In the context of diabetes, butyrate supplementation was shown to increase insulin sensitivity and delay diabetic complications (Gao et al. 2009, Khan & Jena 2015, Roshanravan et al. 2017). More importantly, and to our knowledge, for the first time, our results dissect the mechanism by which butyrate exerts its beneficial effect by inhibiting NADPH oxidase 4-induced ROS production.

**Conclusion**

In conclusion, our study shows that T2DM is associated with a unique microbial signature that is characterized by reduction of B. fragilis and butyrate-forming bacteria. Both bacterial populations secrete metabolites that contribute to the inflammatory state observed with DM. Consequently, mice with T2DM have less butyrate production which impacts its beneficial function as an HDAC inhibitor. Less butyrate resulted in increased activity of HDAC3, which regulates inflammatory pathways and NOX4 expression and results in development of diabetic complications (Fig. 5). Targeting this pathway by probiotics, butyrate supplements, HDAC3 inhibitor, or NOX4 inhibitors represents a potential novel, specific and promising therapeutic approach for the management of T2DM and its complications.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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