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Influence of *Porphyromonas gingivalis* in gut microbiota of streptozotocin-induced diabetic mice

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Abstract

Objectives: Increasing evidence suggests that periodontitis can exacerbate diabetes, and gut bacterial dysbiosis appears to be linked with the diabetic condition. The present study examined the effects of oral administration of the periodontopathic bacterium, *Porphyromonas gingivalis*, on the gut microbiota and systemic conditions in streptozotocin-induced diabetic mice.

Materials and Methods: Diabetes was induced by streptozotocin injection in C57BL/6J male mice (STZ). STZ and wild-type (WT) mice were orally administered *P. gingivalis* (STZPg, WTPg) or saline (STZco, WTco). Feces were collected, and the gut microbiome was examined by 16S rRNA gene sequencing. The expression of genes related to inflammation, epithelial tight junctions, glucose/fatty acid metabolism in the ileum or liver were examined by quantitative PCR.

Results: The relative abundance of several genera, including *Brevibacterium*, *Corynebacterium*, and *Facklamia*, was significantly increased in STZco mice compared to WTco mice. The relative abundances of *Staphylococcus* and *Turicibacter* in the gut microbiome were altered by oral administration of *P. gingivalis* in STZ mice. STZPg mice showed higher concentrations of fasting blood glucose and inflammatory genes levels in the ileum, compared to STZco mice.

Conclusions: Oral administration of *P. gingivalis* altered the gut microbiota and aggravated glycemic control in streptozotocin-induced diabetic mice.

Introduction

Periodontitis is a chronic inflammatory disease that features the periodontal destruction of supporting tissues around the teeth. Recent evidence has been accumulating that periodontitis not only causes teeth loss, but it seems to contribute to the deterioration of systemic conditions. Diabetes is an established risk factor for periodontitis, which is supported by data showing high prevalence, and severe clinical condition of periodontitis in both type 1 and type 2 diabetes mellitus (T1DM, T2DM) (Katagiri et al., 2009; Novotna, Podzimek, Broukal, Lencova, & Duskova, 2015; Taylor et al., 1996). Further, periodontal inflammation negatively affects glycemic control in T2DM patients via insulin resistance, although this is still controversial in T1DM (Graziani, Gennai, Solini, & Petrini, 2017).

Intestinal bacteria play various roles in the host, including inhibition of pathogenic bacterial colonization, food digestion, extraction and synthesis of nutrients and other metabolites, and activation of the immune system, and hence contribute to maintenance of human health (Eckburg et al., 2005). Altered intestinal microbiota in T1DM has been reported as well as in other systemic diseases, such as obesity, atherosclerosis, and non-alcoholic fatty liver disease (Abu-Shanab &

Quigley, 2010; Brown et al., 2011; Karlsson, Tremaroli, Nielsen, & Bäckhed, 2013; Tilg, Cani, & Mayer, 2016; Z. Wang et al., 2011). Several studies have suggested the high abundance of phylum Bacteroidetes/genus *Bacteroides* and/or decreased ratio of Firmicutes to Bacteroidetes as a characteristic of T1DM compared to healthy subjects (de Goffau et al., 2014; Mejía-León, Petrosino, Ajami, Domínguez-Bello, & de la Barca, 2014; Murri et al., 2013), despite conflicting results (Alkanani et al., 2015). In longitudinal studies that observed subjects who seroconverted to become positive for autoantibodies and subsequently progressed to clinical T1DM, the diversity of the intestinal microbiota was decreased after seroconversion (Brown et al., 2011; Giongo et al., 2011; Kostic et al., 2015). The role of the intestinal microbiota in insulin-deficient diabetes has also been investigated using rodent models. Wen and co-workers (Peng et al., 2014; Wen et al., 2008) suggested that alteration of the gut microbiota would not be essential for T1DM development; however, these data indicate that gut microbiota possibly affects the clinical condition of T1DM using NOD mice.

A recent *in vitro* study showed that very low colonization levels of *Porphyromonas gingivalis*, which is a major periodontopathic bacterium, can be triggered to change the composition of oral commensal microbiota and lead to periodontal destruction (Hajishengallis et al., 2011). The gastrointestinal tract commences at the mouth and proceeds to the intestines, thus swallowed periodontal bacteria may proceed along this path. Some oral bacteria can survive in the acidic

environment of the stomach, and subsequently reach the intestines. Qin et al. (Qin et al., 2014) investigated the human gut microbial composition in liver cirrhosis and found a high proportion of taxa known to include species of oral origin. Although the presence of *P. gingivalis* could not be confirmed by PCR in fecal samples in the study, the results indicate that swallowing of periodontopathic bacterium is possibly associated with dysbiosis of the gut microbiota and the induction of subsequent systemic inflammation and metabolic changes.

We hypothesized that dysbiosis of gut microbiota by swallowed bacteria aggravates the clinical condition of T1DM. The influence of insulin-deficient diabetes on the gut microbial composition was confirmed using streptozotocin-induced diabetic mice. Subsequently, the impacts of oral administration of *P. gingivalis* on gut microbiota, glycemic control, and local/systemic inflammations were further investigated in these mice.

Materials and Methods

Mice

Four-week-old male wild-type (WT) C57BL/6J mice were obtained from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). Mice were kept under a 12-h light-dark cycle and were fed food and water *ad libitum*. The mice were randomly divided into two groups. Hyperglycemia was induced by injection with 50 mg/kg streptozotocin (Sigma-Aldrich Co., St. Louis, MO, USA) and citrate buffer

(pH 4.5) intraperitoneally for 5 consecutive days. Three weeks later, mice with greater than 350 mg/dL of fasting blood glucose concentration were considered to have streptozotocin-induced diabetes (STZ). Control mice received an intraperitoneal injection of citrate buffer. All animal experiments were approved (0170331A) by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Oral administration of *P. gingivalis*

Porphyromonas gingivalis ATCC 33277 was anaerobically inoculated in BHI broth medium (supplemented with 5 mg/L of hemin and 50 μg/L of vitamin K_1) for 2 days at 35°C. Bacterial cells in the medium were counted using a bacterial counting chamber, and the concentration was adjusted at 10^9 cells/mL. After centrifugation (8,000 × *g*, 4°C, 10 min), the cells were re-suspended in physiological saline.

Eight-week-old mice were randomly divided into the following four groups: WT mice administered saline (WTco), WT mice administered live *P. gingivalis* (WTPg), STZ mice administered saline (STZco), and STZ mice administered live *P. gingivalis* (STZPg). A total of 10^8 live *P. gingivalis* cells in 100 μL of bacterial suspension was administered to the oral cavity of WT and STZ mice using a feeding needle twice a week for 5 weeks. The control group was administered

100 μL of saline. Two days after the final administration of *P. gingivalis*, the mice (13-week-old) were fasted for 6 h and then euthanized. Samples of venous blood, ileum, liver, and fecal pellets were collected.

Plasma IgG antibody titers in response to *P. gingivalis*

Blood was collected with heparin, and plasma was separated by centrifugation at 6,000 × *g* for 10 min at 4°C. Plasma IgG antibody titers were measured by enzyme-linked immune sorbent assay (ELISA) (D. Wang et al., 2005; D. Wang et al., 2006). Briefly, 96-well microplates were coated with sonicated *P. gingivalis* ATCC 33277 at 10 μg/mL in carbonate buffer, then serially diluted reference positive control plasma (2^5–2^{15}) and diluted plasma (2^{10}) were applied. Subsequently, phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich Co.) was added and developed with phosphatase substrate (Sigma-Aldrich Co.). Optical density at 450 nm was measured by a microplate reader, and antibody titers were calculated in accordance with the method of Wang et al. (D. Wang et al., 2006)

Plasma insulin and cytokine levels

Commercially available kits were used to determine the plasma concentrations of insulin (Ultra Sensitive Mouse Insulin ELISA Kit, Morinaga Institute of Biological Science, Inc., Kanagawa, Japan), tumor necrosis factor-alpha (TNF-α; Mouse TNF-alpha Quantikine ELISA Kit, R&D Systems, Inc., MN, USA), and interleukin-6 (IL-6; Mouse IL-6 Quantikine ELISA Kit; R&D systems Inc.), according to the manufacturer's protocols.

RNA isolation and quantitative real-time PCR analysis

Ileum and liver samples were treated with RNAlater™ Stabilization Solution (Thermo Fisher Scientific Inc., MA, USA) and stored at -80°C until use. Total RNA from the ileum and liver was extracted using NucleoSpin® RNA Kit (Takara Bio Inc., Shiga, Japan) and quantified by measuring absorbance at 260 nm to 280 nm. To synthesize cDNA, 500 ng of RNA was reverse-transcribed using PrimeScript™ RT Master Mix (Takara Bio Inc.), and quantitative polymerase chain reaction (PCR) was conducted on the Thermal Cycler Dice® Real Time System II (Takara Bio Inc.). The mRNA expressions related to inflammation, epithelial tight junctions, glucose metabolism, and fatty acid metabolism were analyzed. The primer sequences for genes are listed in Table S1. SYBR® Premix Ex Taq™ II (Takara Bio Inc.) was used for preparing PCR mixtures, and the PCR thermal profile was set

based on the manufacturer's protocol. Gene expression levels were normalized against *36B4* expression levels. *36b4* has been applied as a reference gene in previous studies that used diabetic mouse models (Katagiri et al., 2016; Ouchi et al., 2010) and is considered to be reliable and suitable as an internal control.

Analysis of gut microbiota by 16S rRNA sequencing

Immediately before euthanizing the animals, fecal pellets were collected and suspended in sterile PBS (1 fecal pellet/2 mL of PBS), and filtered using 100-μm cell strainers (Corning Inc. Corning, NY, USA). To remove debris on the filter, a plastic bar was used with 3 mL of PBS buffer. This was repeated twice, and the filtrate was transferred to a new tube. After centrifuging the filtrate at 5,000 × *g* for 10 min at 4°C, the supernatant was discarded. Collected pellet was washed twice with 5 mL of PBS and subsequently re-suspended in 1 mL of Tris-EDTA (10 mM Tris-HCl and 10 mM EDTA (pH 8.0)). Bacterial DNA was extracted from the fecal samples using enzymatic lysis previously reported by Kim et al. (S. W. Kim et al., 2013).

The V1–V2 region of the 16S rRNA gene was amplified using PCR according to the previous study (S. W. Kim et al., 2013), and the amplicons were sublimated using AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA, USA). After quantification by the Quant-iT

PicoGreen dsDNA Assay Kit (Life Technologies Japan), an equal amount of each PCR amplicon was mixed and sequenced using the MiSeq platform (Illumina Inc.) in accordance with the manufacturer's instructions.

Sequences were analyzed using a previously reported pipeline for pyrosequencing data of the V1–V2 region of the 16S rRNA gene (S. W. Kim et al., 2013). Briefly, 3,000 high-quality 16S reads, with an average quality value >25, were randomly selected from all filter-passed reads of each sample (Supplementary Table S2). We then sorted the selected reads based on average quality values and grouped them into operational taxonomic units (OTUs) through clustering by the UCLUST algorithm (http://www.drive5.com/), employing a 97% identity threshold (Said et al., 2014). For the taxonomy assignment of each OTU, the following publicly available 16S databases were searched for similarity: Ribosomal Database Project v. 10.27, CORE (http://microbiome.osu.edu/), and NCBI genome databases (December 2011). For this, we used the GLSEARCH program. Sequence similarity thresholds for assigning phylum, family, genus, and species levels were set 70, 90, 94, and 96%, respectively. All high-quality 16S V1–V2 sequences analyzed were deposited into the DDBJ/GenBank/EMBL database (accession no. DRA006067).

Alpha-diversity indexes were estimated from the number of observed OTUs, Chao1 species richness estimates, and the Shannon diversity index that measures both species richness and evenness. The diversity of the microbiota was assessed by the weighted UniFrac distance metrics (Said et al., 2014) and visualized with a principal coordinate analysis (PCoA) plot. Permutational analysis of variance (PERMANOVA) analysis was also used to estimate the similarity of the microbiota among four mouse groups.

Statistical analysis

Data distributions were assessed by the Shapiro–Wilk test. ANOVA followed by *t*-tests with Bonferroni correction or Kruskal–Wallis followed by Mann–Whitney U-tests with Bonferroni correction were performed for multiple-group comparisons using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and R software (ver. 3.3.2). $P < 0.05$ was considered statistically significant.

Results

Administration of *P. gingivalis* increases fasting blood glucose and decreases body weight in streptozotocin-induced diabetic mice

Twenty-one STZ-injected mice with high fasting blood glucose (350 mg/dL) and 14 WT mice were randomly divided and administered *P. gingivalis* suspensions (STZPg; n = 11, WTPg; n = 7) or saline (STZco; n = 10, WTco n = 7). Fasting blood glucose concentrations were measured again immediately before sacrifice, and normoglycemic STZ mice were excluded from the present analysis. The final numbers of STZPg and STZco samples analyzed in this study were 9 and 6, respectively.

There were no significant differences in body weight at 8 weeks old (before *P. gingivalis* or saline administration) between WTco and WTPg mice or between STZco and STZPg mice. STZ mice had lower body weights compared to WT mice at 8 weeks. Body weight was significantly lower in STZPg mice than in STZco mice beginning at 3 weeks after administration of the bacterium or saline [$P = 0.009$ (11 weeks), $P = 0.005$ (12 weeks), $P = 0.049$ (13 weeks)]. There were no significant differences in body weight between WTco and WTPg mice during the experimental period (Figure 1a). Before administration of *P. gingivalis* or saline, fasting blood glucose levels were significantly higher in STZco ($P < 0.001$) and STZPg ($P < 0.001$) mice than in WTco and WTPg mice, respectively, but there were no significant differences between STZco and STZPg mice (Figure 1b).

Five weeks after *P. gingivalis* administration, fasting blood glucose levels were significantly higher in STZPg mice ($P < 0.001$) than in STZco mice (Figure 1c). STZco and STZPg mice showed comparable fasting plasma insulin concentrations, which were significantly lower than those of WTco and WTPg mice ($P = 0.048$ and 0.049, respectively). Administration of *P. gingivalis* increased plasma insulin concentration in WT mice (WTPg vs. WTco), although the levels were not statistically significant (Figure 1d). Anti-*P. gingivalis* IgG antibody titers, an indicator of the aggression status of *P. gingivalis,* were higher in mice administered *P. gingivalis* (WTPg and STZPg) than in control mice (WTco and STZco), although the significant difference was only observed between WTPg and WTco mice ($P = 0.024$). There was no significant difference in anti-*P. gingivalis* IgG antibody titers between WTPg and STZPg (Figure 1e).

Evaluation of gut microbiome composition based on 16S rRNA sequences

OTUs based on a 96% genetic difference threshold, the Shannon index and Chao1 estimates were not significantly different among WTco, WTPg, STZco, and STZPg mice (Figure S1a–c). Based on PCoA plots and PERMANOVA test, microbiome diversity did not differ between STZPg-WTPg and STZco-WTco. Further, there were no significant differences when comparing WTco vs. WTPg,

STZco vs. STZPg, and WTco vs. STZco. In contrast, dissimilarity was observed between STZPg and WTPg mice (P = 0.003, Figure S1d).

The compositions of the gut microbiota in WTco, WTPg, STZco, and STZPg mice at the phylum level are summarized in Figure 2a. The relative abundances of major phyla, including Firmicutes and Bacteroidetes, did not differ among the four groups. The relative abundance of phylum Deferribacteres was comparably low in both WTco and STZco mice, but it was overrepresented in *P. gingivalis*-administered (WTPg and STZPg) mice, although a significant difference was only observed in WT (WTco vs. WTPg, P = 0.030) mice (Figure 2b). At the genus level, significant differences in relative abundance were observed for seven genera (Figure 3a). The genus *Lactobacillus* was significantly underrepresented by *P. gingivalis* administration in WT mice (WTPg vs. WTco, P = 0.011). In contrast, *P. gingivalis* administration increased the abundance of this genus in STZ mice (STZPg vs. STZco), although there was no significant difference (Figure 3b). Administration of *P. gingivalis* increased the relative abundance of genus *Turicibacter* in WT mice (WTPg vs. WTco). In contrast, the relative abundance was decreased by *P. gingivalis* administration in STZ mice; hence, significantly lower abundance of *Turicibacter* observed in STZPg mice compared to WTPg mice (P = 0.040, Figure 3c). The relative abundance of genus *Mucispirillum* did not differ between WTco and STZco mice. In contrast, the abundance of the genus became

pronounced in mice who received *P. gingivalis* (WTPg and STZPg), although a significant difference was observed only between WTPg and WTco mice ($P = 0.043$, Figure 3d). The genera *Brevibacterium*, *Corynebacterium*, *Facklamia*, and *Staphylococcus* were rare in WT mice, but overrepresented in STZ mice; hence significantly higher abundance of these genera *Brevibacterium*, *Corynebacterium*, and *Facklamia* observed in STZco compared to WTco ($P = 0.014$, 0.026 and 0.031, respectively). However, a significantly higher abundance of these genera *Brevibacterium*, *Corynebacterium*, *Facklamia*, and *Staphylococcus* were observed in STZPg compared to WTPg ($P =$ 0.006, 0.047, 0.049, and 0.031, respectively). Although the administration of *P. gingivalis* further altered the relative abundance of *Brevibacterium*, *Corynebacterium*, and *Staphylococcus*, there were no statistically significant differences (STZPg vs. STZco) (Figure 3e–h).

Species that showed statistically significant differences in relative abundance among the four groups are listed in Table S3. Highly abundant species (> 0.1%) in the four groups are presented in Figure 4. *P. gingivalis* was not detected in any of the samples. *Facklamia tabacinasalis* was significantly overrepresented in mice with STZ (STZco and STZPg) compared to WT mice (WTco and WTPg), while further alteration was not observed upon administration of *P. gingivalis* in STZ mice. *Clostridium disporicum* was underrepresented in mice administered STZ (STZco and STZPg) compared to WT mice (WTco and WTPg). The relative abundance of *Mucispirillum schaedler* did not

differ between WTco and STZco mice. However, it increased in mice administered *P. gingivalis* (WTPg and STZPg), although a significant difference was observed only between WTPg and WTco mice.

Expression of genes related to tight junctions and inflammation

mRNA expression levels of genes encoding tight junction proteins, including zonula occludens-1 (*ZO-1*), claudin1, and occludin, in the ileum are shown in Figure 5a–c. Although these mRNA levels tended to increase upon administration of *P. gingivalis* (STZPg vs. STZco, WTPg vs. WTco, respectively), there were no significant differences among the four groups.

mRNA expression levels of inflammatory genes in the ileum and liver were also examined (Figure 6a, b). The administration of *P. gingivalis* tended to upregulate the levels of inflammatory genes. In particular, these mRNA levels were significantly higher in STZPg mice than in STZco mice in the ileum ($P < 0.05$, Figure 6a). Expressions of *Tnfa* and *Il6* in the liver were also significantly higher in STZPg mice than in STZco mice ($P = 0.016$ and 0.028, respectively, Figure 6b), while there was no significant difference in mRNA expression levels related to glucose metabolism and lipid metabolism in the liver (Figure 6c).

Plasma levels of inflammatory cytokines

Although plasma TNF-α and IL-6 were measured by ELISA, their levels were below the measurable limits of detection.

Discussion

The various risk factors of T1DM include genetic risk, age, and the presence of autoantibodies. Especially, the gut microbiota is influenced by the host immune condition and changes that correlate with the development of autoantibodies or disease status. In the present study, we used streptozotocin-induced diabetic mice to evaluate the influence of the oral administration of pathogenic bacteria on the gut microbiota of mice with insulin-deficient diabetes without any genetic changes, such as NOD mice. No differences in bacterial diversity and relative abundance of major phyla, such as Firmicutes and Bacteroidetes, were evident between WTco and STZco mice. At the genus level, increased abundances of *Brevibacterium*, *Corynebacterium*, and *Facklamia* were observed, suggesting that diabetes induced by streptozotocin altered the gut microbial composition in mice. Both genera *Brevibacterium* and *Corynebacterium* are from the phylum Actinobacteria, and a few studies have reported higher abundance of taxa belonging to this phylum in patients with T1DM (Brown et al., 2011; de Groot et al., 2017). These bacteria have an ability to produce amino acids, and

alteration of their compositions appeared to be due to the metabolic changes by insulin depletion. Characteristics of *Facklamia* species, including *F. tabacinasalis* (isolated from powdered tobacco (Collins, Hutson, Falsen, & Sjödén, 1999)), are not fully determined, and their roles in gut microbiome are difficult to envision.

The oral administration of *P. gingivalis* resulted in further alteration in the gut microbiota of mice. The relative abundance of genus *Mucispirillum* or *M. schaedleri* from the phylum Deferribacteres increased, irrespective of insulin depletion, rather than by *P. gingivalis* administration. *Mucispirillum* is detected in a variety of mammals, and *M. schaedleri* is known as an abundant inhabitant of the intestinal microbiota in laboratory mice (Sarma-Rupavtarm, Ge, Schauer, Fox, & Polz, 2004). The prevalence of *Mucispirillum* was increased under inflammatory conditions in a rodent model of active colitis (Berry et al., 2012; El Aidy et al., 2014; Hoffmann et al., 2009; Rooks et al., 2014). The characteristics of *M. schaedleri* were recently examined and showed that the bacterium has specialized systems to enable survival under oxidative stress during inflammation. In addition, *M. schaedleri* has the ability to modify the mucosal gene expression in the host (Loy et al., 2017), suggesting that this bacterium might alter the susceptibility to diseases.

P. gingivalis administration induced different quantitative changes in the genera *Lactobacillus*, *Turicibacter*, and *Staphylococcus* in the gut microbiota between STZ and WT mice. The genus *Staphylococcus* is reportedly associated with proinflammatory responses (Edwards et al., 2012). In addition, mRNA expressions of various inflammatory-related genes were increased in the ileum. Therefore, we considered that these alterations were the result of inflammation in the intestinal tracts of *P. gingivalis*-administered STZ mice. A reduction in gut numbers of *Staphylococcus* in new-onset T1DM was observed in a clinical study of Alkanani et al. (Alkanani et al., 2015) However, *Staphylococcus* is enriched in the neonatal gut and is potentially associated with the promotion of anaerobic bacterial growth and/or maturation of the gut microbiota (Marques et al., 2010; Penders et al., 2006). The proportion of the genus *Staphylococcus* might be easily affected by the host immunities and is variable in T1DM. *Turicibacter* is reportedly correlated with the production of butyric acid (Zhong, Nyman, & Fåk, 2015). An increase in butyrate has been associated with improved insulin sensitivity (Vrieze et al., 2012), while a lower abundance of butyrate-producing bacteria was evident in T1DM (Brown et al., 2011; Giongo et al., 2011). In the present study, *P. gingivalis* administration increased the abundance of *Turicibacter* in WT mice, but decreased the abundance in STZ mice. The oral administration of the bacterium might produce negative effects on fasting blood glucose levels by influencing the proportion of this bacterium and insulin sensitivity. In

contrast, the relative abundance of *Lactobacillus* decreased in WTPg mice, but an increasing trend was observed in STZPg mice. *Lactobacillus* spp. are known to enhance the integrity of the intestinal barrier, which may result in the maintenance of immune tolerance and decreased translocation of bacteria across the intestinal mucosa (Lee & Bak, 2011). Further, the proliferation of *Lactobacillus* in the present study might be a homeostatic response in the gut microbiota of STZ mice against *P. gingivalis* aggression. We consider that hyperglycemia and following functional disorders in neutrophils/monocytes caused may influence bacterial growth and alter the composition of gut microbiota in STZ mice.

Aging seems to be associated with diminished intestinal health. A recent mouse model study by van der Lugt (van der Lugt et al, 2018) showed that enrichment of potential pathobionts, including *Desulfovibrio* spp., and a decline of the health-promoting *Akkermansia* spp. and *Lactobacillus* spp. were observed with aging. They also found increased gut permeability in older mice. Increased gut permeability allows passage of microbes and/or microbial products into the internal body, and may cause abnormal immune reactions. While 8 to 13-week-old mice are physically young, the influence of age on the gut microbiome seems to be less or absent in the present study.

Tight junction proteins play a critical role in maintaining the gut barrier function (Tanaka et al., 2015), and several reports have suggested that altered gut microbiota influence the barrier membrane in the gut (K. A. Kim, Gu, Lee, Joh, & Kim, 2012). An increase in intestinal permeability accelerates the infusion of bacterial lipopolysaccharides to the blood stream (Cao et al., 2017) and aggravates glycemic control. Previous studies reported by Yamazaki et al. showed that *P. gingivalis* administration increases intestinal permeability in mice (Arimatsu et al., 2014; Nakajima et al., 2015). In the present study, although the bacterial composition was altered by the administration of streptozotocin or periodontopathic bacteria, these impacts were limited and might not affect the intestinal permeability. The conflicting results might be explained by a difference in the *P. gingivalis* strain and the concentration of the bacterial suspension. In the above-mentioned studies, administered bacterial amounts (*P. gingivalis* W83) per administration seemed high (10^9 CFUs) compared to the present study (10^8 CFUs). In addition, although ATCC 33277 is a major strain type of *P. gingivalis*, several researchers suggested that pathogenesis of this strain is low compared to W83 (Sundqvist, Figdor, Hänström, Sörlin, & Sandström, 1991; Laine & van Winkelhoff, 1998; Grenier & Mayrand, 1987). Interestingly, Yamazaki et al. have reported dramatic alterations of the gut microbiome induced by oral administration of *P. gingivalis* in their three published articles, while the alterations did not necessarily show the same trend. For example, a significantly higher proportion of phylum

Bacteroides in *P. gingivalis* W83-administered C57BL/6 mice was observed in two studies (Arimatsu et al., 2014; Nakajima et al., 2015), while the third report (Kato et al., 2018) showed a significantly higher proportion of this phylum in controls. These results suggested that multiple factors would determine the gut microbial composition and its pathogenicity to induce gut mucosal barrier dysfunction.

Although relative abundance of several genera/species was changed in STZ-induced diabetic mice, it seemed tiny compositional changes for gut microbiome. We used this mouse model for evaluating the influence of insulin deficiency on gut microbiota and subsequent systemic conditions. The present results suggested that the composition of gut microbiota would be regulated by multiple factors, such as diet, growing environment, systemic metabolic conditions, and genetics. The effects of genetic background on the composition of the gut microbiota in mice have been observed (Ericsson et al., 2015). The study examined the gut microbiota of NOD mice, transgenic mice that mimic T1DM, and has shown distinct differences in the microbial composition between NOD and control C57BL/6 mice. Moreover, similarities of gut microbiomes were observed in NOD mice purchased from various animal facilities (Mullaney et al., 2018). Many studies have also described impaired glucose tolerance together with alterations of the gut microbiota composition in T2DM mouse models (Cani et al., 2008; Serino et al., 2012), disturbance of the metabolic system in adipose tissues, and

subsequent inflammation caused by high-fat diet associated with this phenomenon. Systemic metabolic disorders may allow new infection from the oral cavity and lead to a further serious condition. We have reported that oral administration of periodontopathic bacteria in mice with high-fat diet affected glucose/lipid metabolism and aggravated non-alcoholic fatty liver disease (Sasaki et al., 2018; Komazaki et al., 2017). However, Blasco-Baque et al. reported that insulin resistance in the high-fat fed mouse was enhanced by periodontitis caused by alteration of three periodontopathic bacteria in the oral cavity without mainly affecting gut microbiota (Blasco-Baque et al., 2017). It is still unclear and controversial how influx of oral bacteria into the gastrointestinal tract is associated with alteration of the gut microbiota.

Although a robust intestinal epithelial barrier could be maintained after bacterial administration in the present study, oral administration of *P. gingivalis* aggravated fasting blood glucose in STZ mice but not in WT mice. The plasma TNF-α and IL-6 levels were lower than the detection limit, indicating that strong systemic inflammation did not occur in the STZPg mice. In addition, the anti-*P. gingivalis* IgG antibody titers were similar between STZPg and WTPg mice, and the host immune response to *P. gingivalis* was not influenced by streptozotocin treatment. We considered that the aggravation of fasting blood glucose in STZPg mice was mainly due to the increased insulin resistance in the host post administration of the bacterium. The insulin concentration tended to be elevated in WTPg

compared to WTco, although it was not statistically significant. Previous reports showed that oral administration of periodontopathic bacteria diminished glucose tolerance and insulin sensitivity in mice (Arimatsu et al., 2014; Komazaki et al., 2017). Although insulin resistance induced by *P. gingivalis* administration was slight, the amount of insulin secretion in STZ mice reduced drastically, and a pronounced negative effect appeared in the fasting blood glucose control of STZ mice. Although it was difficult to conclusively determine the impact of ingested bacteria on the gut microbiota or systemic conditions, a change in the microbiota composition, especially the genera *Turicibacter* and *Mucispirillum*, seemed to be associated with aggravation of the fasting blood glucose level.

Some researchers demonstrated that repeated oral gavage administration of this bacterium induced alveolar bone resorption (Baker et al., 2000; Hajishengallis et al., 2011; Maekawa et al., 2011). For example, Hajishengallis et al. reported that oral inoculation of 10^9 CFUs *P. gingivalis* ATCC33277 or W50 (given 3 times at 2 day intervals) during six weeks induced periodontal bone loss in SPF mice. In contrast, Arimatsu et al. (Arimatsu et al., 2014) reported that bone resorption did not occur with oral administration of 10^9 CFUs *P. gingivalis* W83 (given 2 times a week for 5 weeks) in C57BL/6N mice. In the present study, the administration interval of the bacterium followed the report of Arimatsu et al., although the bacterial concentration was comparatively lower (10^8 cells). In

addition, *P. gingivalis* suspended in phosphate-buffered saline with 2% carboxymethylcellulose was orally inoculated by a feeding needle in these previous studies. While, the carboxymethylcellulose did not add in the bacterial suspension and administered by a feeding needle in this study. We considered most of bacterial suspension directly flew into the esophagus and would be difficult to remain in oral cavity. Although the oral condition of *P. gingivalis*-administered mice was not evaluated in our study, we inferred there was no or minimal changes in periodontal tissues, including alveolar bone.

In conclusion, our results demonstrate an association between periodontitis and diabetes via oral pathogenic bacterium. An alteration of the gut microbiota was observed after the oral administration of periodontopathic bacteria in mice. The systemic influence attributed to insulin depletion seemed greater, and the slight insulin resistance induced by *P. gingivalis* resulted in an exacerbation of the fasting blood glucose level in streptozotocin-induced diabetic mice.

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Disclosure

The authors declare no conflict of interest in this study.

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Figure legends

Figure 1: Comparison of body weight, fasting blood glucose level, plasma insulin concentration, and anti-*P. gingivalis* IgG antibody titers among WTco, WTPg, STZco, and STZPg mice (n = 6–9).

(a) Body weight. **P* < 0.05 compared to STZco mice. (b) Fasting blood glucose levels in 8-week-old mice (before the administration of saline or *P. gingivalis*). **P* < 0.05. (c) Fasting blood glucose levels in 13-week-old mice (after the administration of saline or *P. gingivalis*). **P* < 0.05. (d) Plasma insulin concentrations in 13-week-old mice after 6 h of fasting. **P* < 0.05. (e) Relative plasma anti-*P. gingivalis* IgG antibody titers in 13-week-old mice. **P* < 0.05.

Figure 2: Compositions of the gut microbiome in WTco, WTPg, STZco, and STZPg mice at the phylum level (n = 6–9).

(a) Mean microbial composition at the phylum level. (b) Phyla (>1.0%) with significant differences in relative abundance among the 4 groups. **P* < 0.05.

Figure 3: Compositions of the gut microbiome in WTco, WTPg, STZco, and STZPg mice at the genus level (n = 6–9).

(a) Mean microbial composition at the genus level. Genera (>1.0%) with significant differences in relative abundance among the 4 groups, (b) *Lactobacillus*, (c) *Turicibacter*, (d) *Mucispirillum*, (e) *Brevibacterium*, (f) *Corynebacterium*, (g) *Facklamia*, (h) *Staphylococcus*. *$P < 0.05$.

Figure 4: Species with high relative abundance (>0.1%) in (a) WTco, (b) WTPg, (c) STZco, and (d) STZPg mice (n = 6–9). *$P < 0.05$ compared to WTco mice. **$P < 0.05$ compared to WTPg mice.

Figure 5: Expression levels of genes encoding tight junction proteins in WTco, WTPg, STZco, and STZPg mice (n = 6–9). (a) *ZO-1*, (b) *Claudin1*, and (c) *Occludin* mRNA expression levels in the ileum. *$P < 0.05$.

Figure 6: Expression levels of genes related to inflammation, glucose metabolism and lipid metabolism in WTco, WTPg, STZco, and STZPg mice (n = 6–9). (a) mRNA expression levels of inflammatory genes in the ileum. (b) mRNA expression levels of inflammatory genes in the liver. (c) mRNA expression levels of glucose metabolism and lipid metabolism in the liver. *$P < 0.05$.

Supplementary material

Table S1: Primers used for quantitative PCR analysis.

Table S2: Summary of Illumina MiSeq reads.

Table S3: Relative abundance ratio of species.

Figure S1: Evaluated of gut microbiome compositions based on 16S rRNA sequence among WTco, WTPg, STZco and STZPg mice.

(a) Number of OTUs (b) Shannon index (c) Chao1 (d) Weighted PCoA

Figure 1

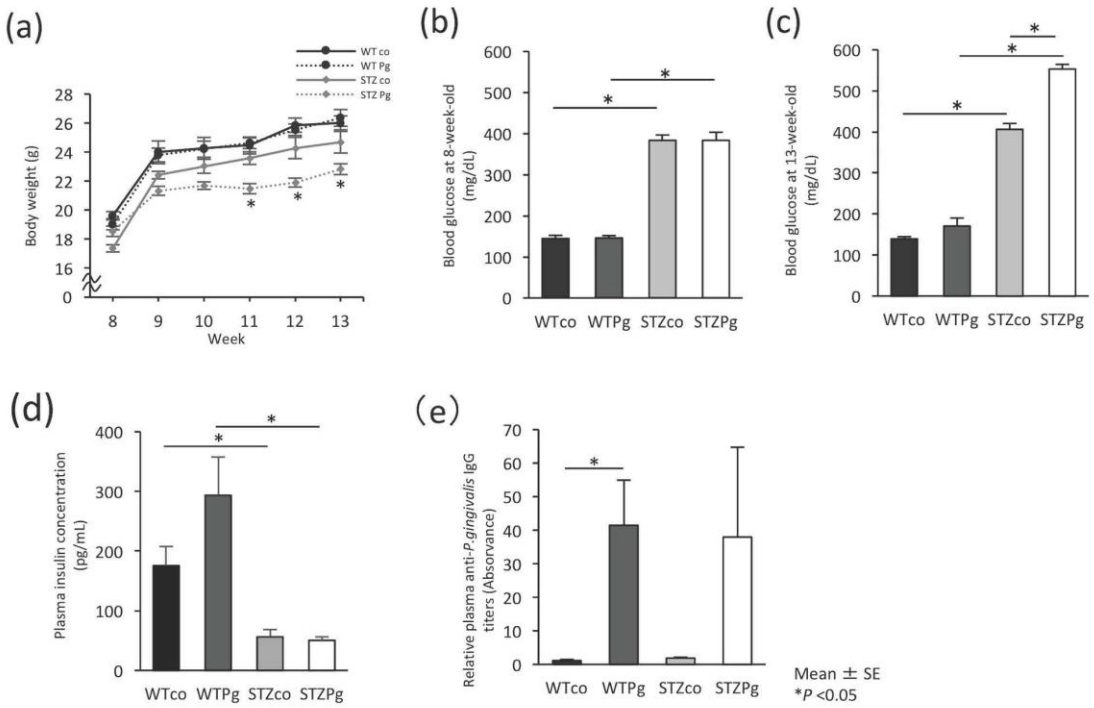


Figure 2

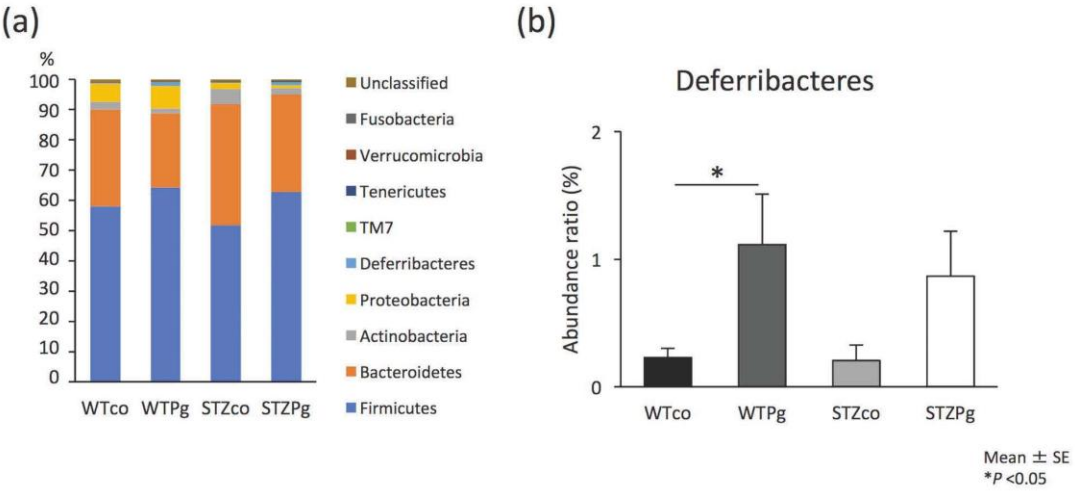


Figure 3

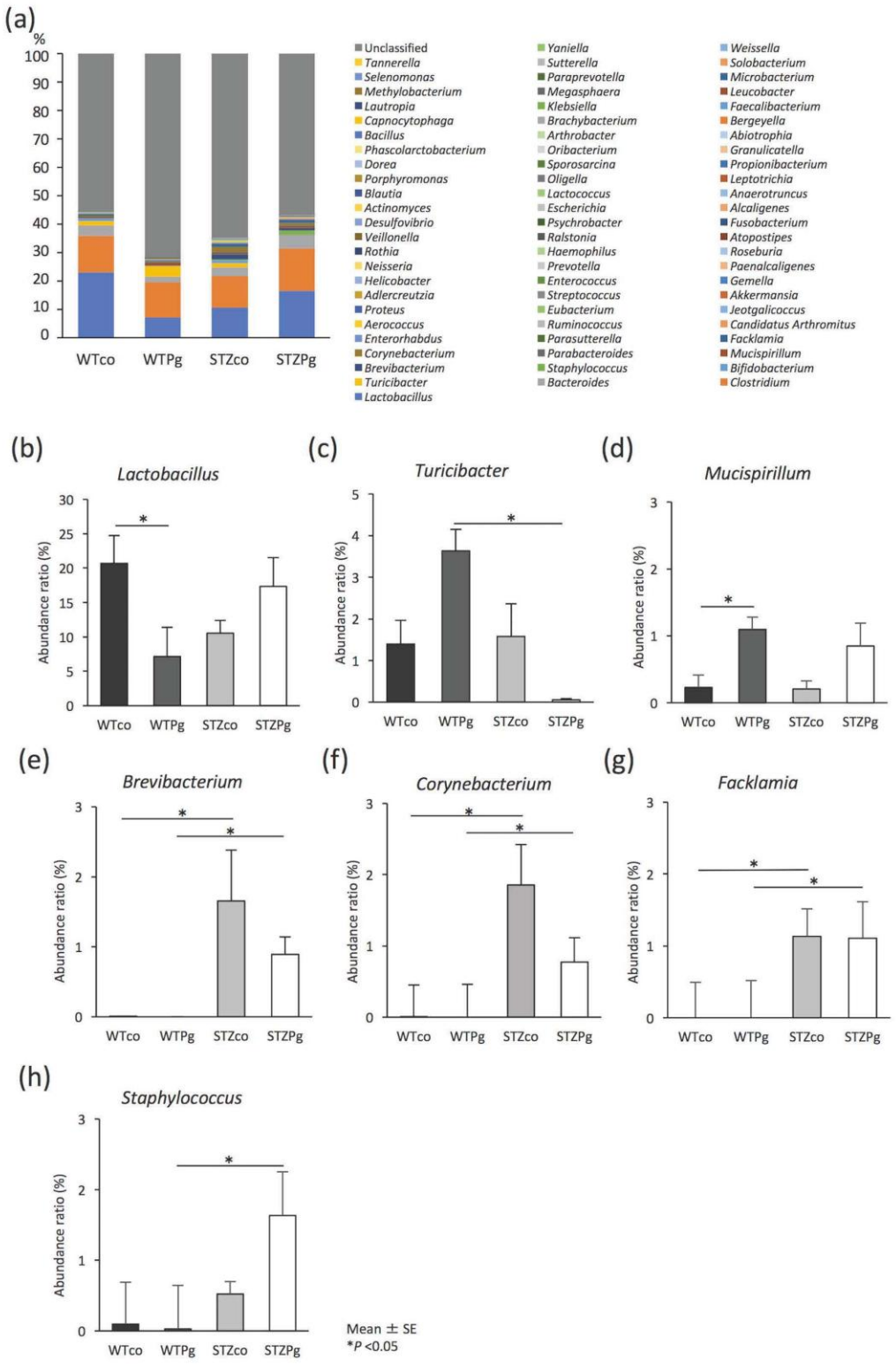
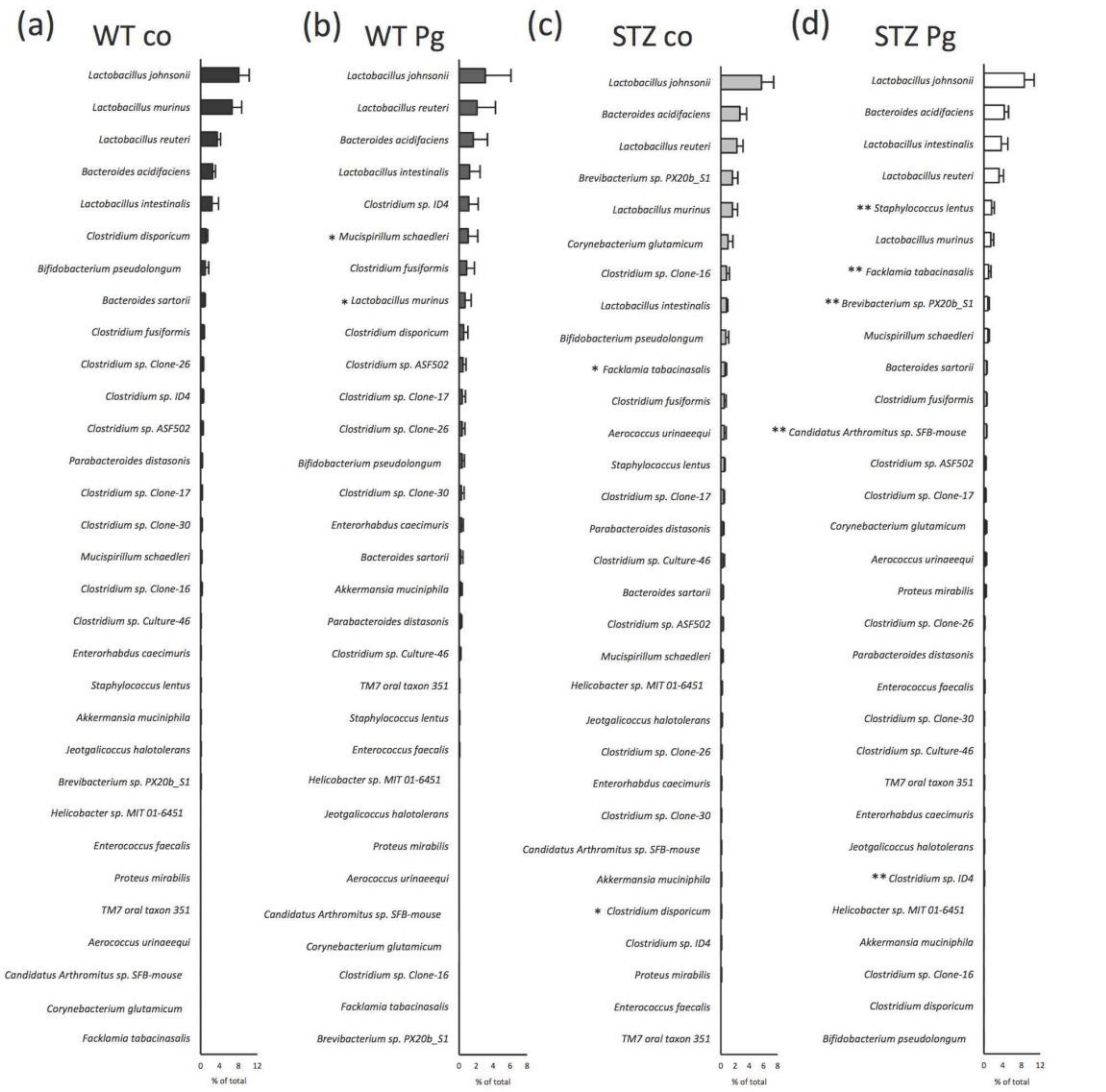


Figure 4



Mean ± SE
*P <0.05 compared to WTco mice
**P <0.05 compared to WTPg mice

Figure 5

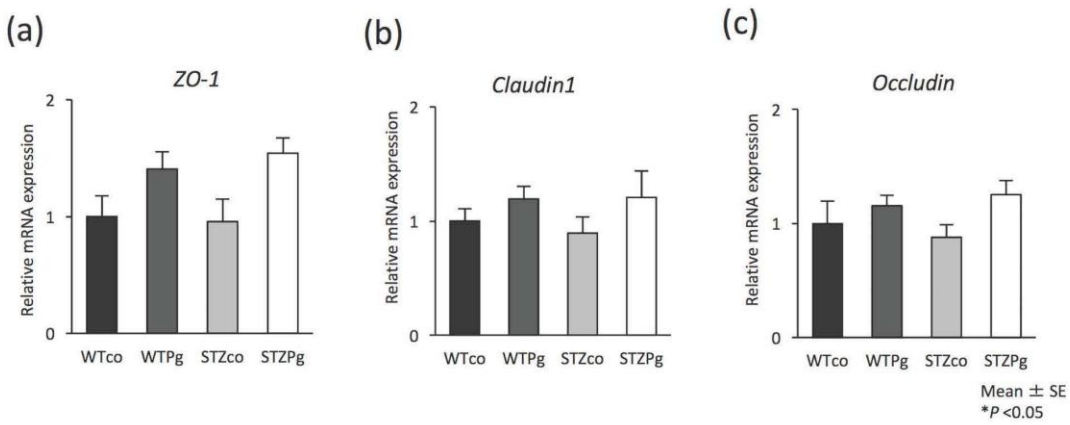


Figure 6