*Porphyromonas gingivalis* induces entero-hepatic metabolic derangements with alteration of gut microbiota in a type 2 diabetes mouse model

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Periodontal infection induces systemic inflammation; therefore, aggravating diabetes. Orally administered periodontal pathogens may directly alter the gut microbiota. We orally treated obese db/db diabetes mice using *Porphyromonas gingivalis* (*Pg*). We screened for *Pg*-specific peptides in the intestinal fecal specimens and examined whether *Pg* localization influenced the intestinal microbiota profile, in turn altering the levels of the gut metabolites. We evaluated whether the deterioration in fasting hyperglycemia was related to the changes in the intrahepatic glucose metabolism, using proteome and metabolome analyses. Oral *Pg* treatment aggravated both fasting and postprandial hyperglycemia (*P* < 0.05), with a significant (*P* < 0.01) increase in dental alveolar bone resorption. *Pg*-specific peptides were identified in fecal specimens following oral *Pg* treatment. The intestinal *Pg* profoundly altered the gut microbiome profiles at the phylum, family, and genus levels; *Prevotella* exhibited the largest increase in abundance. In addition, *Pg*-treatment significantly altered intestinal metabolite levels. Fasting hyperglycemia was associated with the increase in the levels of gluconeogenesis-related enzymes and metabolites without changes in the expression of proinflammatory cytokines and insulin resistance. Oral *Pg* administration induced gut microbiota changes, leading to entero-hepatic metabolic derangements, thus aggravating hyperglycemia in an obese type 2 diabetes mouse model.

Human oral biofilm-forming bacteria cause chronic inflammatory periodontal infection due to a bacterial symbiosis disorder caused by inadequate oral hygiene⁴. *Porphyromonas gingivalis* (*Pg*) causes significant changes in both the amount and composition of normal oral microbiota. *Pg* is a key species causing periodontitis in combination with the other bacteria in periodontal pockets⁴. The main cause of periodontal disease is a dental biofilm composed of periodontal bacteria; the accumulation of this biofilm leads to inflammation and destruction of the periodontal tissues. The previous reports demonstrated that diabetes increases the risk of developing destructive periodontal disease about threefold⁵, and periodontal treatment could be important for effective glycemic management in people with type 2 diabetes⁶. Thus, periodontal disease and systemic diseases especially diabetes have a bidirectional influence on each other. Inflammatory cytokines are chronically overexpressed in accelerated periodontitis, which could exacerbate systemic metabolic diseases⁷–⁹.

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Bacterial cells and their genomic DNA have not been previously identified in fecal specimens, probably because under ad libitum feeding in \( \text{Pg}\)-administered mice was significantly \( (P<0.05) \) higher than that in control mice.

Blood glucose levels were measured at ad libitum feeding. *\( P<0.05 \) as compared with that of the control.

### Results

**Increased fasting and postprandial hyperglycemia following \( \text{Pg} \) treatment.** Blood glucose levels under ad libitum feeding in \( \text{Pg} \)-administered mice was significantly \( (P<0.05) \) higher than that in control mice after 4 weeks of oral \( \text{Pg} \) treatment without any differences in both food intake, body weight and blood glucose at ad libitum feeding during 4-week period between 2 groups (Table 1). Blood glucose levels at both fasting and 2 h after glucose loading were also significantly higher \( (P<0.05) \) in \( \text{Pg} \)-treated mice, compared to control \( \text{db/db} \) mice; however, their fasting hyperinsulinemia and serum triglyceride levels were not different between the 2 treatment groups (Tables 2, 3).

Increased alveolar bone resorption following \( \text{Pg} \) treatment. To assess the severity of the periodontitis, bone loss on the buccal side of the maxillary alveolar bone was measured at five points using \( \mu \text{CT} \) image analysis. \( \text{Pg} \)-treated mice exhibited statistically significant \( (P<0.01) \) alveolar bone resorption, compared to that in control mice (Fig. 1a,b).

### Table 1.

| Group       | Pre  | 1 W  | 2 W  | 3 W  | 4 W  |
|-------------|------|------|------|------|------|
| Food intake/cage (g) | Control 205 ± 3 208 ± 11 217 ± 4 220 ± 26 204 ± 9 |
| n = 4 | \( \text{Pg} \) 205 ± 4 211 ± 10 199 ± 38 208 ± 33 202 ± 38 |
| Body weight (g) | Control 35.3 ± 2.8 38.0 ± 2.0 40.3 ± 2.7 42.4 ± 1.6 43.9 ± 1.8 |
| n = 8 | \( \text{Pg} \) 35.0 ± 2.7 37.7 ± 2.5 40.1 ± 2.5 41.5 ± 2.2 43.0 ± 2.7 |
| Blood glucose (mg/dl) | Control 524 ± 56 615 ± 71 626 ± 119 630 ± 44 651 ± 51 |
| n = 8 | \( \text{Pg} \) 538 ± 54 619 ± 95 698 ± 44 706 ± 66* 735 ± 73* |

### Table 2.

| Group | Blood glucose (mg/dL: fasting) | IRI (μU/mL: fasting) | Triglyceride (mg/dL: ad libitum feeding) |
|-------|--------------------------------|----------------------|----------------------------------------|
| Control | 346 ± 124                       | 32.5 ± 18.7          | 290 ± 101                               |
| \( \text{Pg} \)  | 467 ± 124*                      | 36.5 ± 18.7          | 252 ± 90                                |

The clinical association between periodontal diseases and the poor glycemic control in diabetes is actively investigated. Diabetes is a major risk factor for periodontal disease, with a threefold-higher prevalence of periodontitis in diabetic patients, compared to that in non-diabetic subjects; and poor glycemic control could trigger and worsen periodontitis in diabetes \( (P<0.05) \). Advanced stages of periodontitis may further impair glycemic control in diabetic patients. The mechanisms connecting these conditions has not been elucidated; however, it is speculated that local infection by oral pathogens, and the release of inflammatory cytokines into blood vessels, could explain the systemic effects of periodontal disease.

However, recent studies have proposed that the dissemination of periodontal pathogens into the intestinal tract may induce systemic inflammation, metabolic changes, and fatty liver disease in non-diabetic mice models. Clarifying this requires identification of orally administered periodontal bacteria in fecal specimens. Bacterial cells and their genomic DNA have not been previously identified in fecal specimens, probably because of their rapid digestion by intestinal enzymes.

Here, we studied the presence of \( \text{Pg} \) in fecal specimens at the peptide level, using proteomic analysis of \( \text{Pg} \)-specific peptide fragments, following the oral administration of \( \text{Pg} \) in obese type 2 diabetes mice. Oral bacteria mixed with saliva and food can survive in the acidic stomach environment, and subsequently be transmitted to the intestinal tract with food.

Oral administration of \( \text{Pg} \) in diabetic mice aggravated both fasting and postprandial hyperglycemia, and increased alveolar bone reabsorption. Excessive hepatic gluconeogenesis contributes to hyperglycemia in poorly controlled diabetes \( (P<0.05) \). Therefore, using genomic, proteomic, or metabolomic analyses, we investigated whether \( \text{Pg} \)-administration increased the mRNA and protein expression of hepatic gluconeogenesis-related enzymes, levels of intrahepatic glucose, and that of lipid metabolites in diabetic mice. Previous studies used metagenome analysis to study the changes in the gut microbiota of \( \text{Pg} \)-treated diabetic mice; in this study, we applied metaproteome analysis for the elucidation.

Blood glucose and serum insulin levels at fasting condition, and serum triglyceride levels at ad libitum feeding condition in Porphyromonas gingivalis (\( \text{Pg} \))- and CMC-treated \( \text{db/db} \) mice. Glucose and insulin levels were measured 3 weeks after treatment, and triglyceride levels 4 weeks after treatment. Data are expressed as mean ± standard error of mean. IRI: immunoreactive insulin. *\( P<0.05 \) compared with the control; \( n = 8 \).

Increased alveolar bone resorption following \( \text{Pg} \) treatment. To assess the severity of the periodontitis, bone loss on the buccal side of the maxillary alveolar bone was measured at five points using \( \mu \text{CT} \) image analysis. \( \text{Pg} \)-treated mice exhibited statistically significant \( (P<0.01) \) alveolar bone resorption, compared to that in control mice.
Detection of \textit{Pg}-specific peptides and changes in the microbiome profile of fecal specimens. The metaproteome profiling of the fecal samples was analyzed using the LC–MS/MS-based shotgun proteomics. Approximately 350,000 MS/MS spectra per sample were obtained and were screened against the UniProt database of all putative proteins in the mouse gut metagenome\textsuperscript{21}, the murine UniProt proteome database, and against the proteomes of the food items, and that of \textit{Pg}. The number of peptides identified in the proteome analysis did not differ substantially between the \textit{Pg}- and CMC-treated groups (Table 4). In total, 16,974 unique peptides were identified. Among them, 5576 taxon-specific peptides were matched to 14 phyla of microbes, 2451 peptides to 58 families, and 1626 peptides to 111 genera (Supplementary Table S1).

Six distinct peptides derived from \textit{Pg} were specifically identified in \textit{Pg}-treated \textit{db/db} mice (Table 5). The specific detection of the \textit{Pg}-derived peptides in \textit{Pg}-treated mice were quantitatively assessed using parallel reaction monitoring (PRM) analysis with a synthetic peptide as an internal standard.

| Category | 1st administration | 10th administration |
|----------|--------------------|--------------------|
| Fecal    | \textit{Pg}       | Control          | \textit{Pg}       | Control          |
| Mouse    | 844                | 821               | 1408              | 1276              |
| Bacteria | 5467               | 5061              | 6601              | 6324              |
| Food     | 689                | 759               | 1019              | 1019              |

Table 4. Number of peptides detected during the proteome profiling of the feces of the \textit{Porphyromonas gingivalis} (\textit{Pg})- and carboxymethyl cellulose-treated \textit{db/db} mice. There was no statistical difference in the number of peptides detected, between the \textit{Pg} and CMC control-treated groups.

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in the fecal specimens from Pg-treated, but not in that from the CMC (control)-treated, mice (Fig. 2a,b and Supplementary Figure S1).

The phylum-level microbial composition of the fecal microbiome was profiled based on the metaproteome analysis. Bacteroidetes and Firmicutes dominated the gut microbiota in both Pg-treated and control mice (Fig. 2c). After the 30 day treatment period, the Bacteroidetes population (as a proportion of all bacteria present) was larger; while, the Firmicutes population was smaller in the Pg-treated mice, compared to that in the control group (Firmicutes/Bacteroides: 68%/30% in the Pg-treated group and 50%/49% in the control group, respectively). In addition, the changes in the microbiome were larger following the 10th Pg-treatment period, compared to that after a single treatment of Pg (Fig. 2c). At the family level, Prevotellaceae constituted a higher proportion in the Pg-treated group (27%), compared to that in the control (10%) (Fig. 2d). Prevotella, the most abundant genus in the fecal samples, was present at a higher proportion in the Pg-treated group (37%), compared to that in the control (14%) (Fig. 2e).

Table 5. Distinct peptides of Porphyromonas gingivalis (Pg) derived from the feces of Pg-treated and CMC-treated db/db mice using proteome analysis.

| Peptide sequence | Corresponding protein |
|------------------|-----------------------|
| DVTVEGNSNEAPVQNLGSAVQK | Hemagglutinin A |
| ECVNTVPDPQFPQNLGSAVQK | Hemagglutinin A |
| NDSNTSDYSHENTLQK | DNA-directed RNA polymerase subunit beta |
| LQFTGFDYGPQPGK | Outer membrane protein 40 |
| VAEDIASPVTANAQQVFK | Gingipain R1 |
| VLVDPNYLPDVTAK | Receptor antigen B |

Changes in the intestinal metabolites following Pg treatment. Metabolome analysis of mixed samples of small intestinal tissues and fecal materials showed marked differences in the intestinal metabolites between Pg- and CMC-treated mice. The volcano plots were used to compare the metabolite levels in the fecal specimens of Pg- and CMC-treated mice. Following Pg treatment, the levels of 12 hydrophilic metabolites were significantly elevated; while, that of 35 other metabolites were significantly reduced (Fig. 3). Many end metabolites, such as lactate, phosphoric acid, 3-hydroxybutyric acid, 3-hydroxyisobutyric acid, a valine metabolite, and O-phosphoethanolamine, a metabolite of sphingosine-1-phosphate, were significantly higher in the Pg-treated mice, compared to that in the control mice (Table 6). In contrast, the levels of many amino acids and polyamines were significantly lower in the Pg treated mice, compared to that in the control mice (Table 6).

Changes in the expression of rate-limiting enzymes and the levels of glucose metabolites in the liver of Pg-treated diabetic mice. Enhanced fasting hyperglycemia is regulated by hepatic gluconeogenesis in poorly controlled diabetes. To determine whether the expression of hepatic genes related to gluconeogenesis were upregulated in Pg-treated mice, the expression of phosphoenolpyruvate carboxykinase (Pck1) and Glucose 6 phosphatase (G6pc) was evaluated. Pck1 mRNA expression was significantly higher in Pg-treated mice, compared to that in the control mice (P < 0.05); however, G6pc mRNA expression did not differ significantly between the Pg-treated and CMC-treated mice (Fig. 4a). Expression of Forkhead box protein O1 (Foxo1), a transcription factor in hepatocytes that promotes gluconeogenesis by activating Pck1 and G6pc expression, was also significantly higher in the Pg-treated mice, compared to that in the control. Cytochrome P450 A1 (Cyp 7a1), a rate-limiting enzyme in bile acid biosynthesis, was significantly (P < 0.01) upregulated in the livers of Pg-treated mice, compared to that in the control. Western blot analysis revealed higher levels of PCK1 and FOXO1 protein expression in Pg-treated mice (P < 0.05), compared to that in the control (Fig. 4b). Immunohistochemical analyses indicated upregulated PCK1 and FOXO1 expression in the livers of Pg-treated mice, compared to that in the control (Fig. 4c,d). However, the expression of fatty acid synthase (Fasn) was significantly down-regulated (P < 0.05), while that of acetyl-CoA carboxylase A (Acaca) was lower in the livers of Pg-treated mice, compared to that in the control. The expression of hepatic lipogenesis-related genes, Srebf1 and Srebf2, did not differ significantly between the Pg-treated and control mice (Fig. 4a). mRNA expression of Il-6, Tnf-a, Ccl2, and Cxcl10 were not significantly different between the Pg-treated and control mice (Fig. 4e).

To understand the enhanced fasting hyperglycemia in diabetic mice following oral Pg-administration, the levels of enzymes, glucose metabolites, and lipid metabolites in the liver were quantified through proteomic and metabolomic analyses. There was significant differential expression level between Pg-treated and the control groups, such as a 1.2-fold increase, or more than a 0.83-fold reduction, in Pg-treated mice relative to levels in the control (P < 0.05). Volcano plots of the hepatic glucose metabolites (Fig. 5a) indicated that the levels of 396 proteins, 42 hydrophilic metabolites, and 62 lipids were elevated significantly following Pg-treatment. The
levels of 444 proteins, 6 hydrophilic metabolites, and 12 lipids decreased following the \( Pg \)-treatment (Fig. 5a). Comparative metabolomic analysis revealed that \( Pg \)-administration significantly reduced the glycogen storage in the liver and increased the levels of metabolites related to gluconeogenesis and the tricarboxylic acid cycle (TCA) cycle, such as phosphoenolpyruvic acid (PEP), phosphoenolpyruvic acid (PGA), fumaric acid (FUM), and malic acid (MAL), in the liver (\( P < 0.05 \)) (Fig. 5b). The levels of glycolysis/gluconeogenesis-related proteins, such as PKC1, Tpi1, and Aldoa, were significantly lower (\( P < 0.05 \)) in the treated mice, compared to that in the control (Fig. 5c); while, that of Diat, Pdhb, and Ldha were significantly lower (\( P < 0.05 \)) in the treated mice, compared to that in the control. Changes in the levels of hepatic glucose metabolites and the expression of rate-limiting enzymes of glucose metabolism, which were comparable to enhanced gluconeogenesis in the \( Pg \)-treated diabetic mice as compared with the CMC-treated mice (Fig. 6). There were no significant differences in the levels of fatty acids, glycides, cholesterol, cholesterol esters, phospholipids, and sphingolipids, between the \( Pg \)-treatment and the control groups (Supplementary Fig. S2).

**Discussion**

Specific \( Pg \)-derived peptides were present in the fecal specimens from \( db/db \) diabetic mice after 30 days following oral \( Pg \)-administration. \( Pg \) treatment significantly altered the gut microbiota composition at the phylum, family, and genus levels. The levels of various intestinal metabolites were also altered in the mixed intestinal tissue and feces samples from \( Pg \)-treated mice. These changes were associated with aggravated reabsorption of maxillary alveolar bone and with both fasting and postprandial hyperglycemia. Proteomic and metabolomic analyses revealed that these metabolic changes were associated with the differential expression of the rate-limiting enzymes of glucose metabolism in the liver and with the levels of intrahepatic glucose metabolites, but not with the changes in whole body insulin resistance and the expression of hepatic proinflammatory cytokines.

Our findings confirm the hypothesis that oral bacteria mixed with saliva and food pass through the stomach and reach the intestinal tract. A previous metagenome analysis reported following oral \( Pg \)-treatment, the proportions of Firmicutes and Bacteroides was 55.4% and 38.7%, respectively, in \( Pg \)-treated mice, and 72.8% and 17.0%, in the control mice, respectively. In the present study, we confirmed these findings through proteomic analysis.

*Prevotellaceae* and *Prevotella* populations showed the largest increase in abundance in \( Pg \)-treated mice, compared to that in the CMC-treated mice (Fig. 2d,e). There are close links between the proportion of *Prevotella* and oral and gastrointestinal tract diseases. Therefore, we intend to study the response of *Prevotella* species in the intestinal microbiota to oral administration of periodontal bacteria. The oral administration of a periodontal pathogenic bacterium, *Aggregatibacter actinomycetemcomitans*, alters the gut microbiota composition in a non-diabetic mouse model, with enhanced hepatic fat deposition. This differs from the present results. Furthermore, gut dysbiosis induced by periodontal pathogens is also associated with other biological effects, such as increased intestinal permeability to low molecular weight metabolites produced by invading bacteria. These changes are delivered to the liver, where they could impair glucose tolerance and enhance insulin resistance, while activating the expression of proinflammatory molecules. However, several studies have also indicated that the changes in the levels of specific beneficial metabolites improve whole-body glucose metabolism, the stabilization of intestinal barrier function, or help control obesity. In this study, we observed remarkable changes in the levels of various intestinal metabolites following \( Pg \) treatment, without difference in expression of proinflammatory cytokines in the liver between the two groups.

The liver is crucial for maintaining normal glucose homeostasis, in regulating glycogen synthesis and degradation, glycolysis, and gluconeogenesis, depending on the fasting and postprandial states. In type 2 diabetes with poor glycemic control, the hepatic glucose output is regulated by gluconeogenesis, through changes in the levels of insulin, insulin counter-regulatory hormones, and the supply of gluconeogenic substrates. Oral administration of \( Pg \) upregulated the expression of hepatic gluconeogenesis-related enzymes at the mRNA and protein levels in \( db/db \) mice. Oral administration of periodontal pathogens impairs both glucose tolerance and insulin sensitivity in non-diabetic mice and in streptozotocin-induced diabetic mice. However, in the present study, \( Pg \) treatment did not alter the expression of proinflammatory cytokines in \( db/db \) obese type 2 model mice treated with \( Pg \), as compared to the corresponding expression levels in CMC-treated \( db/db \) mice (Fig. 4e).

Obese type 2 diabetes model mice extensively exhibit insulin resistance and hyperglycemia. Based on the insulin tolerance test (ITT), \( db/db \) diabetic mice in both \( Pg \)- and CMC-treated groups were equally insulin resistant, because of an impaired reduction in the plasma glucose levels at 30–60 min after insulin loading. These results are in line with the lack of further changes in the expression of proinflammatory cytokines in the liver of \( db/db \) mic treated with \( Pg \). There was an increase in the fasting hyperglycemia without changes in the fasting serum insulin levels, indicating that impaired fasting insulin secretion could be a cause for fasting hyperglycemia. The exact molecular mechanisms that induce the progression of fasting hyperglycemia in \( db/db \) mice treated with \( Pg \),...
In addition, consistent with previous reports, PG supply oxaloacetate. Therefore, the altered levels of hepatic glucose metabolites and rate-limiting glucose-treatment; it is possible that these metabolites promote hepatic gluconeogenesis by and pyruvate dehydrogenase B was downregulated, indicating a limited pyruvate flow to the TCA cycle, and treatment. However, the expression of lactate dehydrogenase PCK1 protein and mRNA expression following the liver. Pyruvate is then converted to oxaloacetate via pyruvate carboxylase. Oxaloacetate is then converted to GAP via Tpi1 in the liver (Fig. 6). These metabolites are essential for gluconeogenesis, for the conversion of GAP from the fat cells is converted to dihydroxyacetone phosphate (DHAP) and then glyceraldehyde 3-phosphate to fructose-1,6-bisphosphate (FBP) and DHAP to fructose 1-phosphate (F1P) via aldolase (Aldoa). Both Aldoa and Tpi1 were upregulated in PG-treated mice. Alanine released from skeletal muscle cells is converted to pyruvate via alanine aminotransferase (ALT), and lactate is converted to pyruvate via lactate dehydrogenase (LdhA) in the liver. Pyruvate is then converted to oxaloacetate via pyruvate carboxylase. Oxaloacetate is then converted to PEP via PCK1. Our western blotting, proteomic analyses, and real-time PCR analysis revealed increased levels of PCK1 protein and mRNA expression following PG treatment. However, the expression of lactate dehydrogenase and pyruvate dehydrogenase B was downregulated, indicating a limited pyruvate flow to the TCA cycle, and an increased pyruvate flow to the gluconeogenesis pathway. In addition, the levels of both FUM and MAL were upregulated following PG-treatment; it is possible that these metabolites promote hepatic gluconeogenesis by supplying oxaloacetate. Therefore, the altered levels of hepatic glucose metabolites and rate-limiting glucose-metabolism enzymes following PG-treatment are consistent with enhanced gluconeogenesis in PG-treated db/db mice. In addition, consistent with previous reports, PG treatment reduced glycogen storage in the liver of db/db mice, indicating that the treatment reduced glucose incorporation into glycogen. However, such changes in the levels of hepatic glucose metabolites could be induced by increased plasma insulin-counter regulatory hormone levels, which were not extensively measured in the present study. FOXO1 mRNA expression is increased in the cultured gingival epithelial cells treated with PG. The enhanced FOXO1 expression could modulate multiple keratinocyte functions. Understanding the underlying molecular mechanisms of FOXO1 expression is valuable to be tested in the hepatocytes through the specific PG-derived peptides and the specific PG-induced changes in intestinal metabolites and bacterial flora as one of the future projects. Specifically, it is necessary to evaluate the role of various gut factors in triggering hepatic gene expression using the specifically identified factors in portal vein samples from db/db mice orally treated with PG, in comparison to that in CMC-treated db/db mice.

The findings of this study might not be sufficient to explain the relationship between the change of gut microflora and exacerbation of gluconeogenesis. The altered gene expression in the liver after PG administration could be because of PG or its components being associated with the alternation. Unfortunately, however, it was very difficult to detect PG or its components in the liver of this mouse model. Thus, we alternatively stimulated HepG2 cells (human hepatoma cell line) with PG-derived LPS or human recombinant IL-1β as positive control and examined mRNA expressions of CCL2, CXCL10, and FOXO1 using real time PCR. Interestingly, PG-derived LPS increased the mRNA expression of IL-6, CCL2, CXCL10, and FOXO1 (Supplementary Fig. S3). This suggests that PG or its components may upregulate the expression of both FOXO1 and inflammatory cytokines in the liver. In contrast, the expression of Foxo1 but not IL-6, CCL2 and CXCL10 was increased in the liver (Fig. 4e). We clearly demonstrated that oral PG treatment profoundly alters the gut microbiome profiles at the phylum, family, and genus levels. Particularly, Prevotella showed the largest increase in abundance following PG treatment. Moreover, there were profound metabolite changes in the intestinal and fecal samples. Taken together, we speculate that specific metabolites generated by the changes in the intestinal microbiota may affect the expression of Foxo1 and the enhancement of gluconeogenesis without affecting inflammatory cytokine levels in the liver.
Volcano plots of intestinal metabolites in fecal specimens of diabetic mice orally treated with *Porphyromonas gingivalis* (*Pg*) compared with those for CMC-treated mice.

Figure 3. Volcano plot of fold change in the levels of intestinal hydrophilic metabolites in fecal specimens of *db/db* diabetic mice orally treated with *Porphyromonas gingivalis* (*Pg*) or CMC (control). Blue points represent a significant increase or decrease.

of the gut factors or specific metabolites triggering the expression of hepatic genes could be an important aspect to be investigated in future studies using the portal vein samples from the *Pg*-administered *db/db* mice.

In conclusion, we identified *Pg*-specific peptides in fecal specimens of obese type 2 diabetes model mice orally administered with *Pg* for 30 days. Proteomic analysis revealed that the presence of *Pg* in the intestine significantly altered the gut microbiome profile at the phylum, family, and genus levels. *Pg* treatment significantly altered the levels of intestinal end metabolites, several amino acids, and polyamines. *Pg* treatment aggravated both fasting and postprandial glucose levels, and increased the levels of gluconeogenesis-related metabolites and enzymes. However, there was no increase in the expression of proinflammatory cytokines in the liver and insulin resistance in the *Pg* treated *db/db* mice, which are typical characteristics in obese type 2 diabetes model mice.

Methods

Animals. Forty-one male *C57BLKS/Jlar- + Lepr/db + Lepr/db* (*db/db*) mice aged 6 weeks were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were maintained under controlled temperature (23 ± 2 °C) and light–dark cycle with free access to food and water, and fed a regular chow diet (5.1% fat, 55.3% carbohydrate, 23.1% protein; MF Oriental Yeast Co., Ltd., Tokyo, Japan). After acclimatization for a week, the mice were randomly assigned to *Pg*-treated (*n* = 20) and CMC-treated (*n* = 21) groups. The bacterial load administered in the mouse periodontitis model was based on Baker et al.38. *Pg* and CMC were administered orally through a plastic tube, with 10⁹ CFU *Pg* mixed with 4% CMC (for the *Pg*-treatment), or only CMC (for the control), every 3 days for 30 days. The experiment was divided into four administrating sessions with 4–6 animals in each group. The food intake in *db/db* mice was measured for 6–11 consecutive weeks. The animals and the amount of food in the cage were weighed once a week. After anesthesia using mixed anesthesia (Domitor, 0.75 mg/kg body weight; Midazolam, 4 mg/kg; and Butorphanol Tartrate, 5 mg/kg), blood was collected from inferior vena cava and liver samples were excised and harvested for the following experiments. Maxillae were removed from euthanized *db/db* mice and fixed using 4% paraformaldehyde for 48 h.

The oral glucose tolerance test was performed following overnight (10 h) fasting, 21 days after the initial *Pg* treatment. Fasting glucose levels were measured, and mice were orally administered with 2 g glucose/kg body weight. The intraperitoneal insulin tolerance test was conducted with intraperitoneal insulin injections (5 units/kg body weight), 26 days after the initial *Pg* treatment. Blood glucose levels were measured at 0, 30, 60, and 120 min after insulin administration.
To determine insulin levels, blood samples were collected from the inferior vena cava of anesthetized mice. Serum insulin levels were determined using the insulin ELISA kit (FujiFilm Wako Shibayagi Corporation, Gunma, Japan), following the manufacturer’s instructions.

All animal experiments were performed according to the protocols approved by the institutional animal care and use committees of Osaka University Graduate School of Dentistry (permit number: 27–022-0). In addition, all methods were performed in compliance with the ARRIVE guidelines.

**Bacterial culture.** The *Pg* strain (ATCC33277) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown at 37 °C for 24 h in an anaerobic box chamber (Mitsubishi Gas Chemical Company, Inc. Tokyo, Japan) with AnaeroPack-Anaero anaerobic gas generator (Mitsubishi Gas Chemical Com-

| Downregulation | *Pg*/Control | *P* value | Upregulation | *Pg*/Control | *P* value |
|----------------|--------------|-----------|--------------|--------------|-----------|
| Spermine       | 0.010        | 0.00087   | Fructose 1-phosphate | 2.7          | 0.01357   |
| Tyramine       | 0.050        | 0.00520   | 3-Hydroxybutyric acid | 2.3          | 0.03464   |
| Cytosine       | 0.060        | 0.00310   | 3-Hydroxyisobutyric acid | 2.3          | 0.02438   |
| Tryptophan     | 0.090        | 0.00180   | Fructose 1-phosphate | 2.2          | 0.01465   |
| Histidine      | 0.090        | 0.02100   | O-Phosphoethanolamine | 1.9          | 0.00931   |
| Lysine         | 0.10         | 0.00170   | Phosphoric acid     | 1.6          | 0.03399   |
| Tyrosine       | 0.11         | 0.00020   | Lactic acid         | 1.5          | 0.01121   |
| Ornithine      | 0.14         | 0.00560   |
| Arginine       | 0.16         | 0.00210   |
| Lactose-meto   | 0.17         | 0.02200   |
| Asparagine     | 0.18         | 0.02900   |
| Lactose        | 0.18         | 0.04200   |
| Phenylalanine  | 0.22         | 0.00310   |
| N-Acetyl glutamine | 0.24    | 0.00138   |
| Gluconic acid  | 0.25         | 0.00580   |
| Galacturonic acid | 0.26     | 0.01400   |
| Arabinol       | 0.27         | 0.00280   |
| Dihydroxyacetone | 0.28    | 0.00890   |
| Allantoin      | 0.29         | 0.01900   |
| Pyruvic acid   | 0.30         | 0.02800   |
| Octopamine     | 0.32         | 0.00210   |
| Leucine        | 0.32         | 0.01400   |
| Methionine     | 0.36         | 0.02000   |
| Isoleucine     | 0.37         | 0.01600   |
| Glutamine      | 0.40         | 0.04900   |
| Isoleucine     | 0.42         | 0.00037   |
| Pantothenic acid | 0.42     | 0.00330   |
| Arabinose-meto | 0.43         | 0.00410   |
| Glutamic acid  | 0.44         | 0.04300   |
| Valine         | 0.47         | 0.03300   |
| Fructose-meto  | 0.47         | 0.03900   |
| Fructose       | 0.49         | 0.04000   |
| Ornithine      | 0.50         | 0.02300   |
| 5-Oxoproline   | 0.57         | 0.02200   |
| Cysteine       | 0.58         | 0.02100   |

Table 6. Intestinal metabolites with significantly different expression in the *Porphyromonas gingivalis* (*Pg*)- and carboxymethyl cellulose-treated *db/db* mice. Intestinal metabolite levels were measured using metabolome analysis of mixed intestinal tissue and fecal material specimens. Data are expressed as mean ± standard error of mean; *n* = 3.
(a) Immunohistochemical analysis of hepatic Pck1 expression in Pg- and CMC-treated diabetic mice

(b) Immunohistochemical analysis of hepatic FOX01 expression in Pg- and CMC-treated diabetic mice

(c) Immunohistochemical analysis of hepatic Foxo1 expression in Pg- and CMC-treated diabetic mice

(d) Immunohistochemical analysis of hepatic Acaca expression in Pg- and CMC-treated diabetic mice

(e) Immunohistochemical analysis of hepatic Srebfl expression in Pg- and CMC-treated diabetic mice

(f) Immunohistochemical analysis of hepatic Srebfl2 expression in Pg- and CMC-treated diabetic mice

* p<0.05
** p<0.01
**Figure 4.** Effects of oral *Porphyromonas gingivalis* (Pg) administration on mRNA and protein expression in the liver of *db/db* mice. (a) mRNA expression of *Pck1*, *G6pc*, *Foxo1*, *Cyp7a1*, *Fasn*, *Acaca*, *Cpt1*, *Sreb1*, and *Sreb2*. mRNA expression was determined using quantitative RT-PCR and was normalized against the expression of 18S rRNA mRNA. Target gene expression in Pg-treated mice was normalized against the target gene expression in CMC-treated mice. Prior to the observation, the intraclass correlation for the evaluation of bone loss was examined. One examiner evaluated the same teeth points on different days. The resulting intraclass correlation coefficient was 0.86. (b) Western blot analysis of PCK1, G6PC, and FOXO1 in liver tissues from *db/db* mice treated with Pg or CMC for 30 days. β-actin was used as the loading control. The bar graphs on the right show densitometric quantification of the amounts of PCK1, G6PC, and FOXO1, relative to that in the control. n = 4; *P* < 0.05 versus control. (c, d) PCK1 (c) and FOXO1 (d) detection in liver tissues of Pg- or control-treated mice. Left column: Paraffin-embedded sections stained with hematoxylin and eosin (H&E). Right column: Immunohistochemical detection. Scale bars: 100 μm. (e) Comparison of the relative gene expression of proinflammatory cytokines (*Il-6*, *Tnf-a*, *Ccl2*, and *Cxc10*) in the liver tissues of Pg- and control-treated *db/db* mice. The data were normalized and analyzed as described for (a). n = 20–21. Pck1, Phosphoenolpyruvate carboxykinase 1; G6pc, Glucose-6-phosphatase; Foxo1, Forkhead box protein O1; Cpt1c, Carnitine palmitoyltransferase 1c; Fasn, Fatty acid synthase; Acaca, Acetyl-Coenzyme A carboxylase alpha; Sreb1, Sterol regulatory element-binding transcription factor 1; Sreb2, Sterol regulatory element-binding transcription factor 2; Il-6, Interleukin 6; Tnf-a, Tumor necrosis factor-α; Ccl2, C-X-C motif ligand 2; Cxc10, C-C motif chemokine ligand 10.

**Quantification of alveolar bone resorption.** Morphometric analysis of the buccal alveolar bone resorption was performed using an R_mCT2 3D micro X-ray computed tomography system designed for use with scanned images of laboratory animals (Rigaku, Tokyo, Japan). An examiner blinded to the experimental groups measured the linear distances of the cemento-enamel junction (CEJ) from the alveolar bone crest (ABC) using the 3D image analysis software TRI/3D-BON (RATOC System Engineering Co., Ltd., Tokyo, Japan). Buccal-side maxillary alveolar bone loss (ABL) was measured from the cemento-enamel junction (CEJ) to alveolar bone crest (ABC) at five points: (1) distobuccal regions for first maxillary molar (M1); (2) mesiobuccal and (3) distobuccal regions for second maxillary molar (M2); and (4) mesiobuccal and (5) distobuccal regions for third maxillary molar (M3), after 30 days following the treatment with Pg or CMC in *db/db* mice. Distance between the CEJ and the ABL was measured at five sites in the apical direction using WinROOF software version 7.4 (https://www.mitani-visual.jp/products/#image_analyse_ismasurement) (Mitani Corporation, Fukui, Japan), and total value of five points on the μCT image was defined as the alveolar bone loss, were compared in Pg or CMC-control treated groups. Prior to the observation, the intraclass correlation for the evaluation of bone loss measurements was examined. One examiner evaluated the same teeth points on different days. The resulting intraclass correlation coefficient was 0.86.

**Real-time PCR.** Total RNA from the mouse liver was extracted using a RNeasy lipid tissue mini kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions. cDNA was synthesized from 100 ng total RNA using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). PCR was performed using the ABI 7300 real-time PCR system with the Power SYBR Green PCR master mix (both from Applied Biosystems), according to the manufacturer's protocol. To control for the variations in the amount of DNA available for PCR, target gene expression in each sample was standardized based on the expression of an endogenous control. The sequences of the primers used are provided in Supplementary Table S2.

**Protein analysis.** Total proteins were extracted from the frozen liver tissues using the T-PER tissue protein extraction reagent (Thermo Fisher Scientific Inc., Waltham, MA), and used for western blotting. Immunoblotting was performed using the following primary antibodies: PCK1 (1:1000; ab28455, Abcam, Toronto, Canada), G6PC (ab83690; Abcam), FOXL1 (1:000; 2880, Cell Signaling Technology, Danvers, MA), and β-actin (A5216, Sigma-Aldrich Co., St. Louis, MO), and incubated with anti- rabbit HRP-conjugated secondary antibody (1:10,000; NA934, GE Healthcare, Chicago, IL, USA) anti- mouse HRP (1:10,000; NA931, GE Healthcare). Immunoreactive bands were visualized using ECL (Thermo Fisher Scientific).

**Histology.** Liver tissues, excised from mice after 30 days following the oral administration of Pg and CMC, were fixed using 4% paraformaldehyde for 48 h and embedded in paraffin. Samples were then deparaffinized, rehydrated, and washed with PBS. The tissue sections were cut at 4 μm thickness with LEICA RM2245 (Leica Microsystems, Wetzlar, Germany) and stained with hematoxylin and eosin (H&E). For immunohistochemistry, samples were embedded in paraffin, sectioned, and stained with rabbit anti-PCK1 (ab2845, 0.4 μg/mL; Abcam) and analyzed as described for (a).
(a) Volcano plots of hepatic glucose metabolites in diabetic mice orally treated with Pg compared with those for CMC-treated mice.

(b) Changes in hepatic glycogen content and substrate relating to gluconeogenesis in Pg- and CMC-treated diabetic mice.

(c) Changes in hepatic rate-limiting enzymes to regulate gluconeogenesis and glycolysis in Pg- and CMC-treated diabetic mice.

(d) Hepatic glycogen synthesis and degradation and glycogen content in Pg- and CMC-treated db/db mice.
Lys-C endopeptidase and trypsin, as previously described. The extracted protein was subjected to reductive alkylolation, followed by successive digestion with acids and nucleotides; and with liquid chromatography with a pentafluorophenyl propyl column coupled with a high-resolution tandem mass spectrometer (IC/MS/MS) for anionic polar metabolites, such as organic acids and nucleotides; and with liquid chromatography with a pentfluorophenyl propyl column coupled with a high-resolution tandem mass spectrometer (PFPP-LC/MS/MS) for cationic polar metabolites, such as amino acids. The levels of free fatty acids (FAs) and cholesterol esters (ChEs) in the liver samples were quantified using supercritical fluid chromatography with a C18 column coupled with triple quadrupole mass spectrometry (C18-SFC/MS/MS). The levels of other lipids—phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatic acid (PA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), monoacylglycerol (MG), diacylglycerol (DG), triacylglycerol (TG), sphingomyelin (SM), cholesterol, ceramide (Cer), and hexosylceramide (HexCer)—were quantified using SFC with a diethylamine (DEA) column coupled with triple quadrupole mass spectrometry (DEA-SFC/MS/MS). The levels of other lipids—phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatic acid (PA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), monoacylglycerol (MG), diacylglycerol (DG), triacylglycerol (TG), sphingomyelin (SM), cholesterol, ceramide (Cer), and hexosylceramide (HexCer)—were quantified using SFC with a diethylamine (DEA) column coupled with triple quadrupole mass spectrometry (DEA-SFC/MS/MS). Details regarding sample preparation and the analytical conditions for the analysis of the hydrophilic and hydrophobic metabolites are provided as Supplementary Methods.

**Statistical analysis.** All data are presented as mean ± SEM. Differences in body weight, food intake, and blood glucose levels between the Pg and CMC (control) groups were analyzed using one-way ANOVA with Tukey’s post hoc test. All other comparisons between the two groups were analyzed using an unpaired t-test. Differences were considered statistically significant at P < 0.05.
Figure 6. Schematic presentation of the levels of hepatic glucose metabolites and the expression of rate-limiting enzymes of glucose metabolism in $\text{Pg}^-$ and CMC-treated diabetic mice. Fold changes in the glucose metabolites and enzymes involved in intrahepatic glucose metabolism in the $\text{Pg}^-$ treated mice, relative to that in the control. Red circles and lines: significant increases. Blue circles and lines: significant reductions. Metabolites that varied substantially and significantly between the $\text{Pg}^-$ treated mice and the control were identified using these criteria: log$_2$ fold change >|0.26| and $P<0.05$, and $P<0.01$. 2KG, 2-Ketoglutaric acid; 6PG, 6-Phosphogluconic acid; Ace CoA, Acetyl-CoA; BPGA, 1,3-Bisphosphoglycerate; Cit, Citric acid; DHAP, Dihydroxyacetone phosphate; F1P, d-Fructose 1-phosphate; F6P, d-Fructose 6-phosphate; Frc, d-Fructose; G1P, d-Glucose 1-phosphate; G6P, d-Glucose 6-phosphate; GAP, Glyceraldehyde 3-phosphate; Glc, d-Glucose; Isocit, Isocitric acid; Lac, Lactic acid; MAL, Malic acid; Oxalosuccinate; Suc, Succinic acid; Suc CoA, Succinyl-CoA; UDP-Gal, UDP-alpha-d-galactose; UDP-Glc, Uridine 5'-diphosphate glucose; AGL, Amylo-1,6-glucosidase, 4-alpha-glucanotransferase; Aldoa, Fructose-bisphosphate aldolase A; Dlat, Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial; Pdhb, Pyruvate dehydrogenase E1 component subunit beta; Fh1, Fumarate hydratase-1; Gpi1, Glucose-6-phosphate isomerase 1; Gys2: Glycogen [starch] synthase 2; Lidha, L-lactate dehydrogenase; Pck1, Phosphoenolpyruvate carboxykinase; Pgm2, Phosphoglucomutase-2; Idh3a, Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial; Sdha, Succinate dehydrogenase [ubiquinone] subunit alpha, mitochondrial; Sdhl, Succinate dehydrogenase (ubiquinone) cytochrome b560 subunit; Acly, ATP citrate (pro-S)-lyase; Tpi1, Triosephosphate isomerase 1.
Author contributions

Y.K., S.A., and N.S. are co-first authors and contributed equally to this work. Y.K. devised the study. Y.K., S.A., Y.S., Y.N., Y.I., and N.S. designed the study protocol. Y.K., Y.N., S.T., and S.H. performed mouse and bacterial growth experiments. Y.S., H.U., R.Y., N.S., and Y.I. performed proteomic experiments. S.A., M.T., Y.I., and T.B. performed metabolomic experiments. R.Y., S.A., U.H., N.S. and Y.I. led the data integration for the bioinformatics analysis and interpreted the analytical outcomes in close collaboration with Y.K., Y.N., Y.I., T.B., T.N., S.Y., A.K., and S.M. Y.K., S.A., N.S., and Y.I. wrote the manuscript. Y.I., T.B., A.K., and S.M. revised the paper. All authors approved the final version of the manuscript. S.M. is responsible for the integrity of all data.

Competing interests

The authors declare no competing interests.

Additional information

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