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Continuous glucose monitoring reveals periodontitis-induced glucose variability, insulin resistance, and gut microbiota dysbiosis in mice

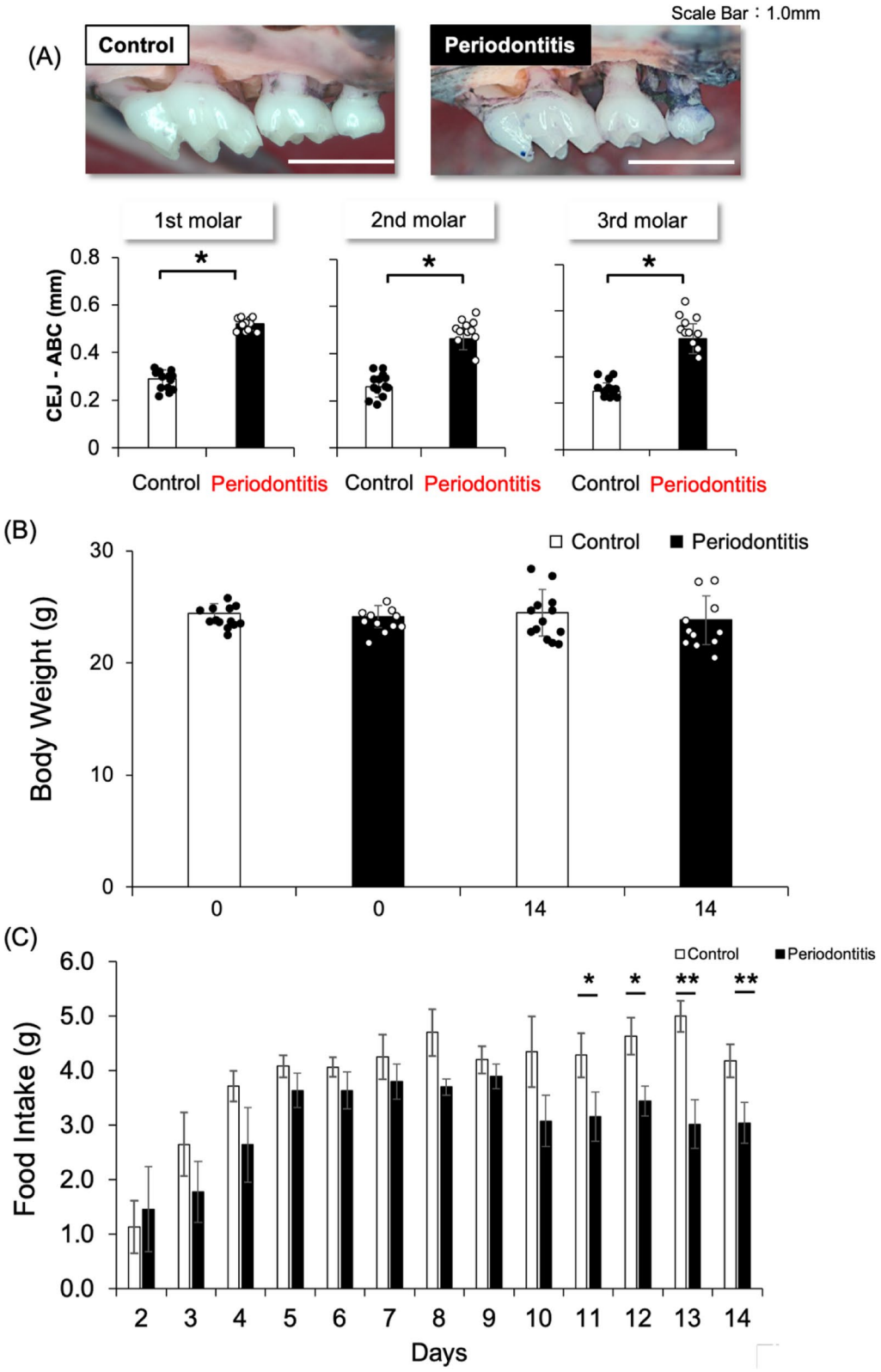
Moyuka Kubota-Takamori[1], Kazuhiro Omori[2]✉, Chiaki Kamei-Nagata[3], Fumiko Kiyama[1], Takayuki Ishii[1], Masaaki Nakayama[4], Kazuyoshi Gotoh[5], Kimito Hirai[3], Yuki Shinoda-Ito[2], Keisuke Okubo[3], Shin Nakamura[2], Atsushi Ikeda[3], Tsugumichi Saito[6], Jun Wada[7] & Shogo Takashiba[2]

Diabetes mellitus (DM) management has advanced from self-monitoring blood glucose (SMBG) to continuous glucose monitoring (CGM), which better prevents complications. However, the influence of periodontitis—a common DM complication—on glucose variability is unclear. This study examined glucose variability in mice with periodontitis using CGM. Periodontitis was induced in 9-week-old male C57BL/6J mice via silk ligatures around the upper second molars. Glucose levels were monitored over 14 days with CGM, validated by SMBG. On day 14, samples were collected to assess alveolar bone resorption and serum levels of tumor necrosis factor-α (TNF-α), insulin, and amyloid A. Glucose tolerance test (GTT) and insulin tolerance test (ITT) were conducted to evaluate insulin resistance. Gut microbiota diversity was also analyzed. By day 10, mice with periodontitis exhibited higher mean glucose levels and time above range than controls. On day 14, serum insulin and amyloid A levels significantly increased, while TNF-α remained unchanged. GTT and ITT indicated insulin resistance. Microbiota analysis showed reduced alpha- and altered beta-diversity, with decreased *Coprococcus spp*. and increased *Prevotella spp*., linking dysbiosis to insulin resistance. Periodontitis disrupts glucose regulation by promoting insulin resistance and gut microbiota imbalance, leading to significant glucose variability.

Keywords Continuous glucose monitoring, Periodontal disease, Insulin resistance, Chronic inflammation, Gut flora

Diabetes mellitus (DM) is a lifestyle-related disease characterized by persistent hyperglycemia due to insulin deficiency or resistance[1], and its prevalence continues to rise each year. As of 2021, approximately one in ten adults aged 20–79 worldwide – equating to 537 million people – have diabetes[2]. Poor glycemic control in diabetes patients can lead to three major complications: diabetic retinopathy, nephropathy, and neuropathy[3]. Periodontitis, a condition that impacts oral health, is referred to as the "sixth most common complication" of diabetes[4]. Severe periodontal inflammation adversely affects glycemic control, creating a harmful cycle. The onset and progression of these complications can greatly diminish patients' quality of life (QOL), highlighting

[1]Department of Pathophysiology-Periodontal Science, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700-8525, Japan. [2]Department of Pathophysiology-Periodontal Science, Faculty of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700-8525, Japan. [3]Department of Periodontics and Endodontics, Division of Dentistry, Okayama University Hospital, Okayama 700-8558, Japan. [4]Department of Oral Microbiology, Faculty of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700-8525, Japan. [5]Department of Medical Laboratory Science, Okayama University Graduate School of Health Sciences, Okayama 700-8558, Japan. [6]Department of Health & Sports Sciences, Faculty of Education, Tokyo Gakugei University, Koganei 184-8501, Tokyo, Japan. [7]Department of Nephrology, Rheumatology, Endocrinology and Metabolism, Faculty of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700-8558, Japan. ✉email: kazu@okayama-u.ac.jp



the importance of prevention and management strategies. In today's aging society, the incidence of periodontitis is increasing as more older adults retain their natural teeth[5]. As a result, the potential for periodontitis to exacerbate the pathogenesis of DM has become more evident.

Traditionally, a specific group of pathogens known as the red complex, which includes *Porphyromonas gingivalis* (Pg), *Treponema denticola*, and *Tannerella forsythia*, has been considered the primary cause of periodontal disease[6]. However, a new hypothesis suggests that these bacteria are non-pathogenic. Instead, they act as keystone pathogens that disrupt the balance of microbial communities and drive disease progression[7]. Among these pathogens, *P. gingivalis* has attracted particular attention due to its role as a key pathogen. Research

◀**Fig. 1**. Comparison of Alveolar Bone Resorption, Body Weight and Food Intake. (**A**) Palatal side of the right maxillary first to third molars is shown. The extent of alveolar bone resorption in each tooth in the respective groups is shown (control, n = 13; periodontitis, n = 11). CEJ, cementoenamel junction; ABC, alveolar bone crest. Scale bar: 1.0 mm. Statistical analysis was performed using the Mann–Whitney *U* test. (**B**) A comparison of the body weight between control and periodontitis groups at the beginning and end of the experiment is presented in the graph (control: n = 13, periodontitis: n = 11). Statistical analysis was performed using the Mann–Whitney *U* test. (**C**) A comparison of the daily food intake between control and periodontitis groups is shown in the graph. Statistical analysis was performed using a two-way analysis of variance and Bonferroni's multiple comparison test. *, $p < 0.05$; **, $p < 0.01$. Open bar, control; Solid bar, periodontitis.

has shown that Pg produces virulence factors such as fimbriae (lineage hairs) and lipopolysaccharide (LPS), which modulate the host immune response and trigger inflammation. Furthermore, it forms biofilms through interactions with other microorganisms, disrupting the oral microbiome. This process shifts oral health from eubiosis (a balanced, healthy microflora) to dysbiosis (a state of microbial imbalance)[7]. Recent findings have underscored the broad effects of periodontal disease on systemic health. Traditionally, it is believed that periodontal pathogens and inflammatory mediators spread through the bloodstream, contributing to systemic conditions like cardiovascular disease and diabetes[6]. However, emerging evidence indicates that periodontal disease also influences gut microbiota. It is now suggested that dysbiosis within the gut environment may exacerbate systemic inflammatory and metabolic diseases. Therefore, maintaining eubiosis in both the oral and gut microbiomes appears to be a critical factor in preserving overall systemic health[7].

For individuals with DM, effective daily blood glucose management is crucial for preventing the development or progression of complications. Traditionally, self-monitoring of blood glucose (SMBG) using specialized blood collection and monitoring devices has been the standard method. However, this approach places a significant burden on patients and does not effectively capture the variability in blood glucose levels between measurements. Recently, there has been a notable shift toward continuous glucose monitoring (CGM). This technology involves continuously measuring glucose levels in subcutaneous interstitial fluid using a dedicated sensor. Unlike SMBG, which provides only isolated data points, CGM automatically measures glucose levels at regular intervals, typically every 15 min. This allows for the identification of daily variability in blood glucose levels, which are displayed as a line graph. As a result, CGM enables the timely detection of conditions that were previously difficult to identify, such as latent hypoglycemia and hyperglycemia. Given its advantages in improving glucose-monitoring accuracy and reducing patient burden, CGM has emerged as a widely adopted method for blood glucose control in patients with DM worldwide. Most studies examining the interaction between DM and periodontitis have traditionally relied on SMBG and used HbA1c levels as indicators to evaluate the impact of periodontal inflammation on blood glucose control[8,9]. However, recent clinical research suggests that HbA1c levels alone are insufficient to assess the risk of diabetic complications accurately. Instead, monitoring diurnal variations in blood glucose levels through CGM is equally essential[10–12]. Recent clinical studies have demonstrated that non-surgical periodontal therapy in patients with type 2 DM not only improves HbA1c levels but also reduces glycemic variability, as assessed by CGM metrics such as time-in-range (TIR)[13]. These observations suggest a potential association between periodontal inflammation and daily variability in blood glucose levels. However, the impact of periodontal inflammation on diurnal blood glucose variation, including its underlying mechanisms, remains unclear.

The null hypothesis of this study was that periodontitis does not affect diurnal blood glucose variation, while the alternative hypothesis was that periodontitis disrupts diurnal blood glucose variation. The primary outcome was diurnal blood glucose variation, assessed using CGM, including average glucose levels, TIR, time above range (TAR), and time below range (TBR). In this study, we investigated the impact of periodontal inflammation on diurnal variability in blood glucose levels using a mouse ligature-induced periodontitis model equipped with a CGM sensor.

Results

Assessment of alveolar bone resorption

To evaluate the progression of periodontitis in ligature-treated mice, alveolar bone resorption was assessed 14 days after periodontitis was induced. No tooth loss was observed in either group at the time of sample collection or during the process of bone sample collection. Furthermore, alveolar bone resorption was significantly more pronounced in the periodontitis group than in the control group for all tooth types (control, n = 13; periodontitis, n = 11, $p < 0.0001$, Fig. 1A).

Comparison of body weight and food intake

Body weight and food intake were compared between the control and periodontitis groups. No significant differences in body weight were observed between the two groups at the start of the experiment or 14 days after periodontitis induction (control, n = 13; periodontitis, n = 11; Fig. 1B). However, food intake was significantly lower in the periodontitis group than in the control group from day 10 after induction (control, n = 13; periodontitis, n = 11, day 11: $p = 0.023$; day 12: $p = 0.011$; day 13: $p = 0.001$; day 14: $p = 0.004$; Fig. 1C).

Evaluation of CGM measurements compared to SMBG

The SMBG values and corresponding CGM measurements of the control and periodontitis groups were compared. No significant differences were found between SMBG values and contemporaneous CGM measurements in either group (n = 13, Fig. 2).

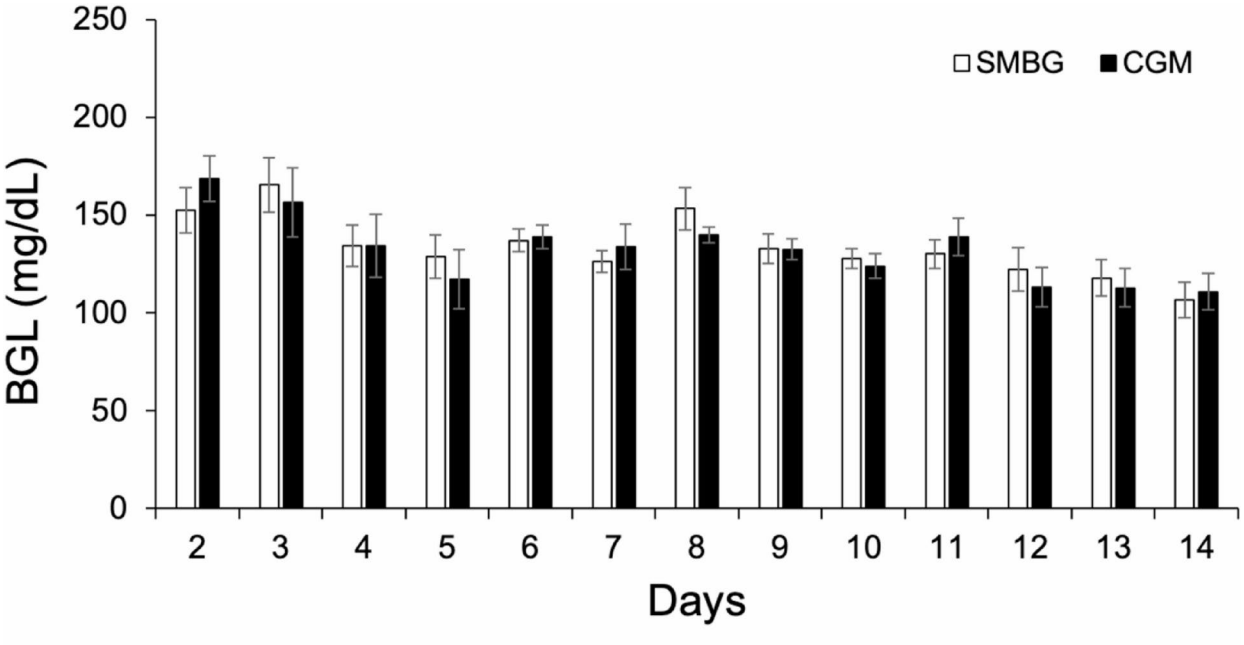


Fig. 2. Comparison of SMBG and CGM Sensor Readings. The graph presents a comparison of SMBG and CGM sensor readings taken simultaneously. SMBG, self-monitoring of blood glucose; CGM, continuous glucose monitoring; BGL, blood glucose level.

Focusing on day 14 of periodontitis induction, it was observed that the periodontitis group exhibited disrupted diurnal variations in blood glucose levels compared to the control group (A representative analytical example of diurnal blood glucose variation; Fig. 3A). Comparison of CGM data between the control and periodontitis groups showed a significant increase in mean daily blood glucose levels beginning on day 10 after periodontitis induction (control, n = 13; periodontitis, n = 11, day 10, $p = 0.043$; day 11, $p = 0.061$; day 12, $p = 0.018$; day 13, $p = 0.003$; day 14, $p = 0.001$; Fig. 3B). Comparison of CGM data between the control and periodontitis groups revealed a significant increase in TAR in the periodontitis group beginning on day 10 after periodontitis induction (control, n = 13; periodontitis, n = 11, day 10, $p = 0.004$; day 11, $p = 0.002$; day 12, $p = 0.001$; day 13, $p = 0.005$; day 14, $p = 0.003$; Fig. 3C). The actual CGM data for both the control group and the periodontitis group over the 14-day observation period are presented as Supplementary Fig. 1. The correlation between the percentage of bone resorption and TAR on day 14 was analyzed using Spearman's rank correlation coefficient. In the control group, no correlation was observed ($p = -0.0764$, $p = 0.8027$). In contrast, the periodontitis group showed a moderate negative correlation ($p = -0.4690$, $p = 0.1478$), although it did not reach statistical significance. Fisher's z transformation revealed no significant difference between the two groups ($z = -0.79$, $p = 0.43$, two-tailed).

Comparison of serum insulin concentrations

Serum insulin concentrations were compared between control and periodontitis groups. Fourteen days after the induction of periodontitis, the serum insulin levels in the periodontitis group were significantly higher than those in the control group (control, n = 13; periodontitis, n = 11, $p = 0.0414$; Fig. 4A). The correlation between the percentage of bone resorption and insulin levels was analyzed using Spearman's rank correlation coefficient. In the control group, a weak negative correlation was observed ($p = -0.3755$, $p = 0.2043$). In the periodontitis group, the correlation was weaker and not statistically significant ($p = -0.1376$, $p = 0.6907$). Fisher's z test indicated no significant difference in the strength of correlation between the two groups ($z = -0.54$, $p = 0.59$, two-tailed).

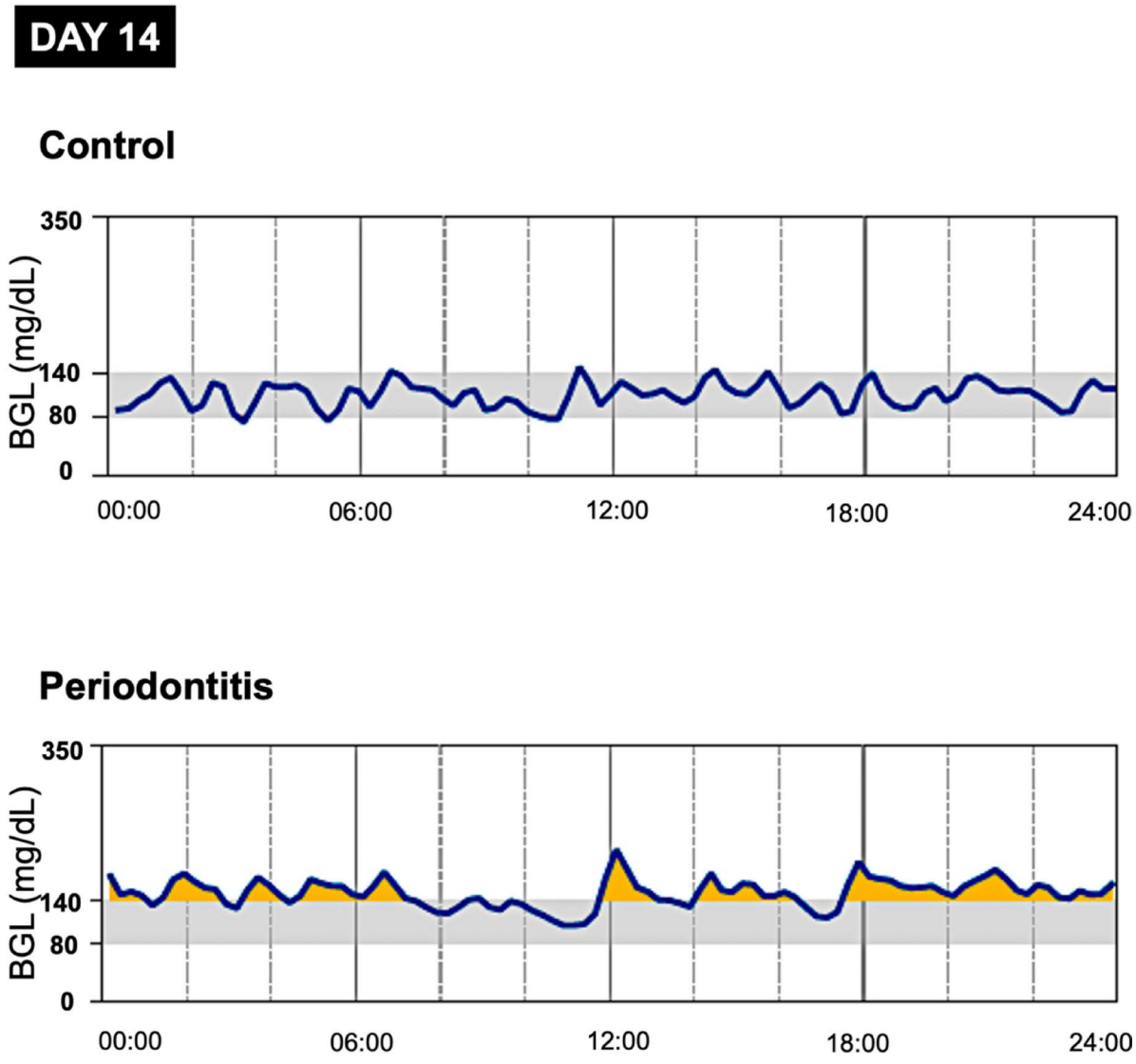
Comparison of serum amyloid A (SAA) concentrations

SAA concentrations were measured to evaluate acute systemic inflammation in mice. Fourteen days after the induction of periodontitis, SAA levels were significantly higher in the periodontitis group than in the control group (control, n = 13; periodontitis, n = 11, $p < 0.001$; Fig. 4B). The correlation between the percentage of bone resorption and serum SAA levels was assessed using Spearman's rank correlation coefficient. In the control group, a weak negative correlation was observed ($p = -0.3755$, $p = 0.2043$), whereas in the periodontitis group, the correlation was weaker and not statistically significant ($p = -0.1376$, $p = 0.6907$). No significant difference in the strength of correlation between the two groups was detected by Fisher's z test ($z = -0.54$, $p = 0.59$, two-tailed).

Comparison of TNF-α concentration in serum

To assess systemic inflammatory responses in mice with periodontitis, serum levels of pro-inflammatory cytokine TNF-α were measured and compared. No significant differences in TNF-α production were observed between the periodontitis and control groups (control, n = 13; periodontitis, n = 11, $p > 0.05$; Fig. 4C).

(A)



Measured values

	Ave. of DGL (mg/dL)	Stable (%)	TBR (%)	TAR (%)
Control	109	92	4	4
Periodontitis	149	30	0	70

Fig. 3. Diurnal Variation of CGM. (**A**) The actual CGM levels of control and periodontitis groups during the day are presented in graphs and summarized in a table. BGL, blood glucose level; DGL, daily glucose level; TAR, time above range; TBR, time below range. (**B**) Mean daily blood glucose levels and (**C**) percentages of TAR of control and periodontitis groups during the experimental period are presented (control: n = 13, periodontitis: n = 11). BGL, blood glucose level; DGL, daily glucose level; TAR, time above range. Statistical analysis was performed using a two-way analysis of variance and Bonferroni's multiple comparison test. *, $p < 0.05$; **, $p < 0.01$.

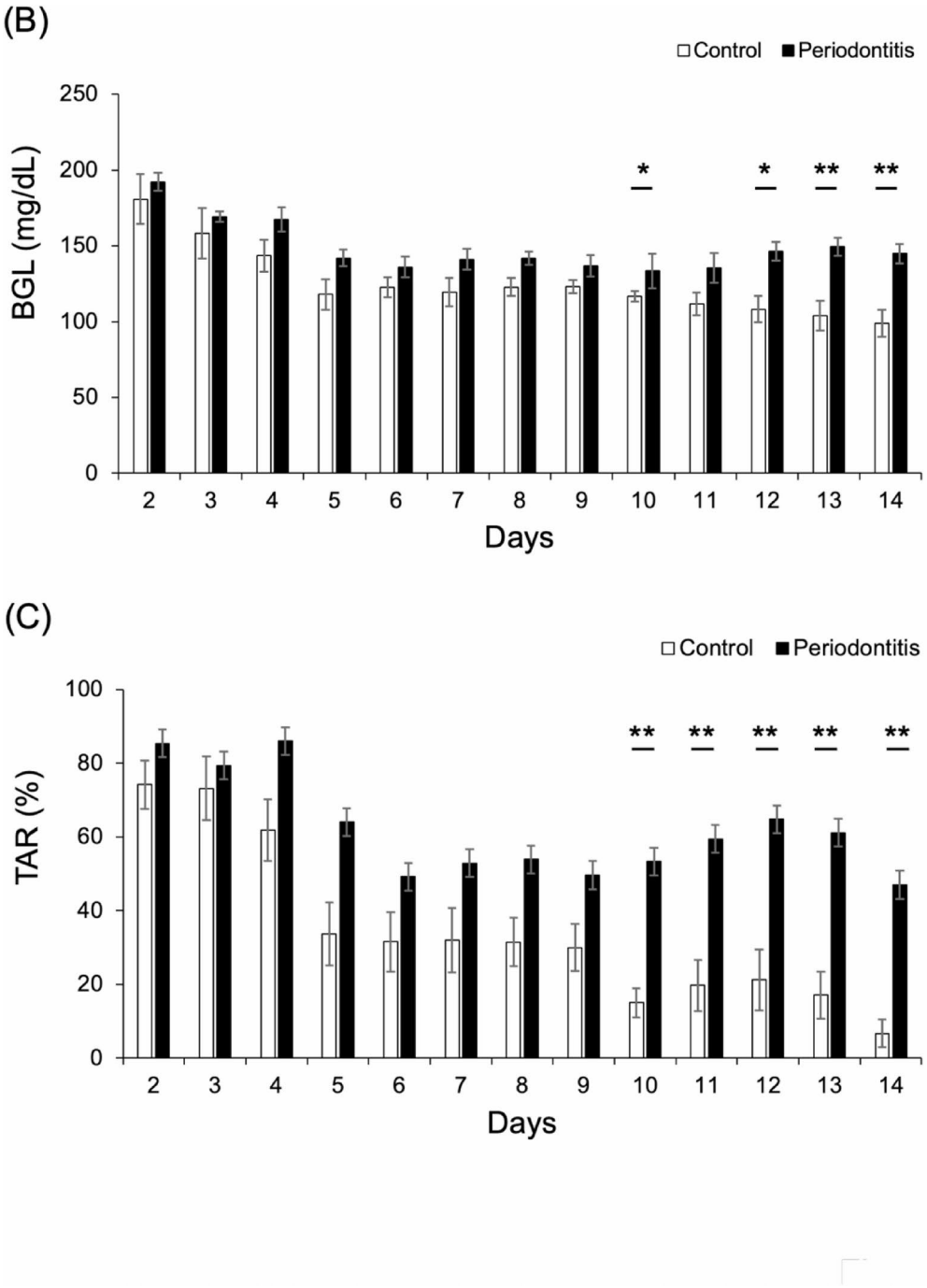


Fig. 3. (continued)

Comparison of GTT and ITT

Impairment of glucose metabolism and insulin resistance were evaluated using GTT and ITT. In the GTT, blood glucose levels in the periodontitis group were significantly higher at 120 min after glucose administration (control, n = 4; periodontitis, n = 4, $p = 0.0286$; Fig. 5A). Similarly, in the ITT, blood glucose levels were significantly reduced in the periodontitis group 30 min after insulin administration (control, n = 4; periodontitis, n = 4, p = 0.0286; Fig. 5B). In the control and periodontitis groups, serum insulin levels were measured before and 30 min after the glucose tolerance test. As a result, in the periodontitis group, serum insulin levels were measured 30 min after glucose administration and were found to have significantly increased (control, n = 4; periodontitis, n = 4, $p = 0.0084$; Fig. 5C).

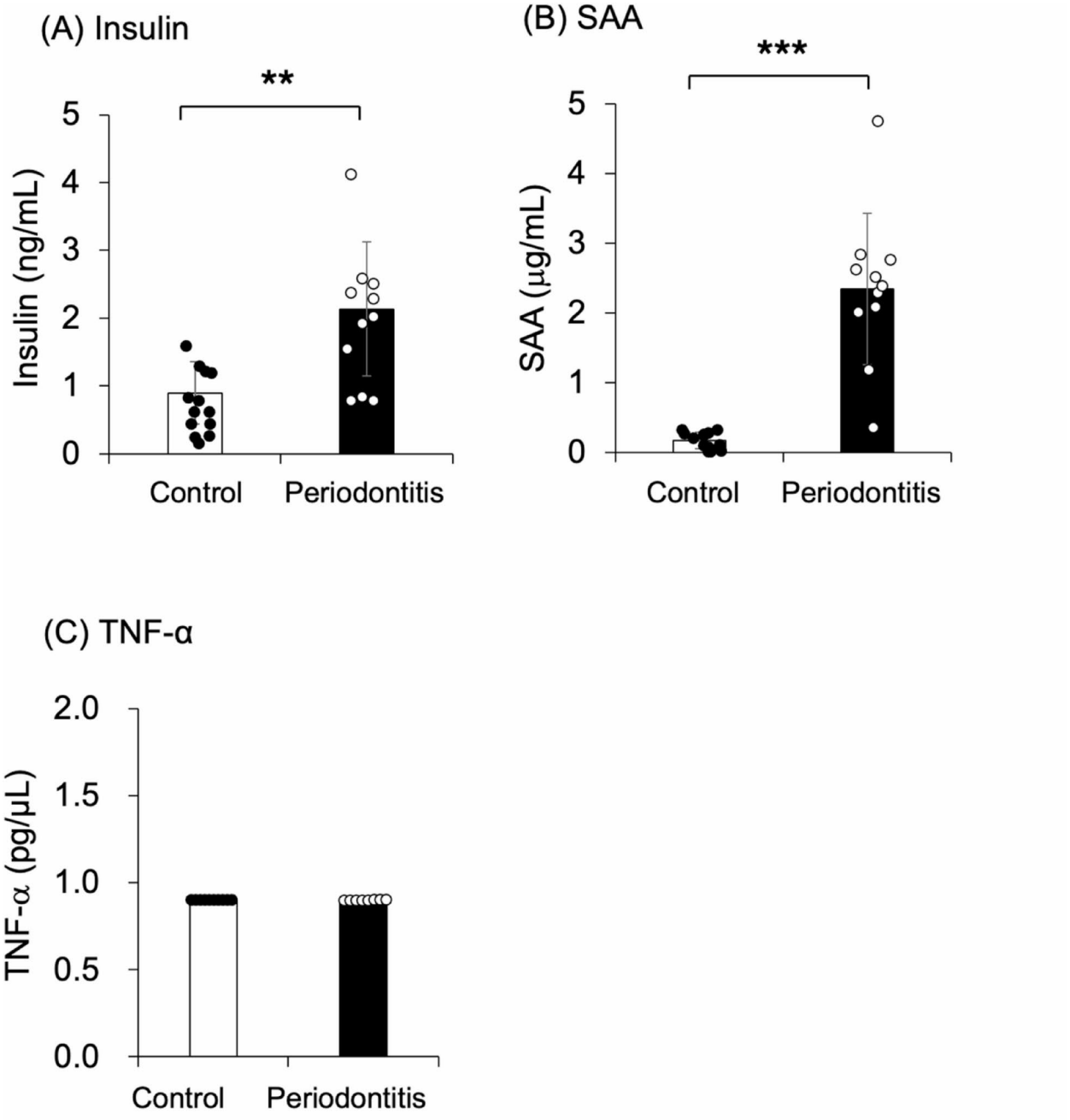


Fig. 4. Changes in Concentrations of Serum Insulin, Inflammatory Cytokines, and Serum Amyloid A The concentrations were measured using ELISA. (**A**) serum insulin concentration: control: n = 13, periodontitis: n = 11; (**B**) SAA: control: n = 13, periodontitis: n = 11; (**C**) TNF-α: control: n = 13, periodontitis: n = 11). SAA, serum amyloid A. Statistical analysis was performed using the Mann–Whitney *U* test. **, $p < 0.05$. ***, $p < 0.001$. Note: TNF-α was above detection limits (TNF-α, 0.1 pg/μL).

Comparison of microbiome analysis

We analyzed the gut microbiome of fecal samples collected 14 days after periodontitis induction. First, alpha-diversity, measured using Faith's PD, showed reduced diversity in the periodontitis group, although the difference was not statistically significant (control, n = 4; periodontitis, n = 4, $p = 0.386$; Fig. 6A). Next, beta-diversity analysis revealed a significant difference in gut microbiota composition between the control and periodontitis groups, as determined by the Unweighted UniFrac distance (control, n = 4; periodontitis, n = 4, $p = 0.027$; Fig. 6B). LEfSe analysis identified bacterial taxa that significantly differed between the two groups (Fig. 6C). In the periodontitis group, *Prevotella* (LDA score = 3.8, $p = 0.0433$), *Lactobacillus* (LDA = 3.6, $p = 0.0209$), and *Escherichia* (LDA = 3.5, $p = 0.0139$) were significantly enriched. In contrast, *Coprococcus* (LDA = –3.9, $p = 0.0202$), *Clostridium* (LDA = –3.4, p = 0.0472), and the family *Mogibacteriaceae* (LDA = –3.2, $p = 0.0433$) were enriched

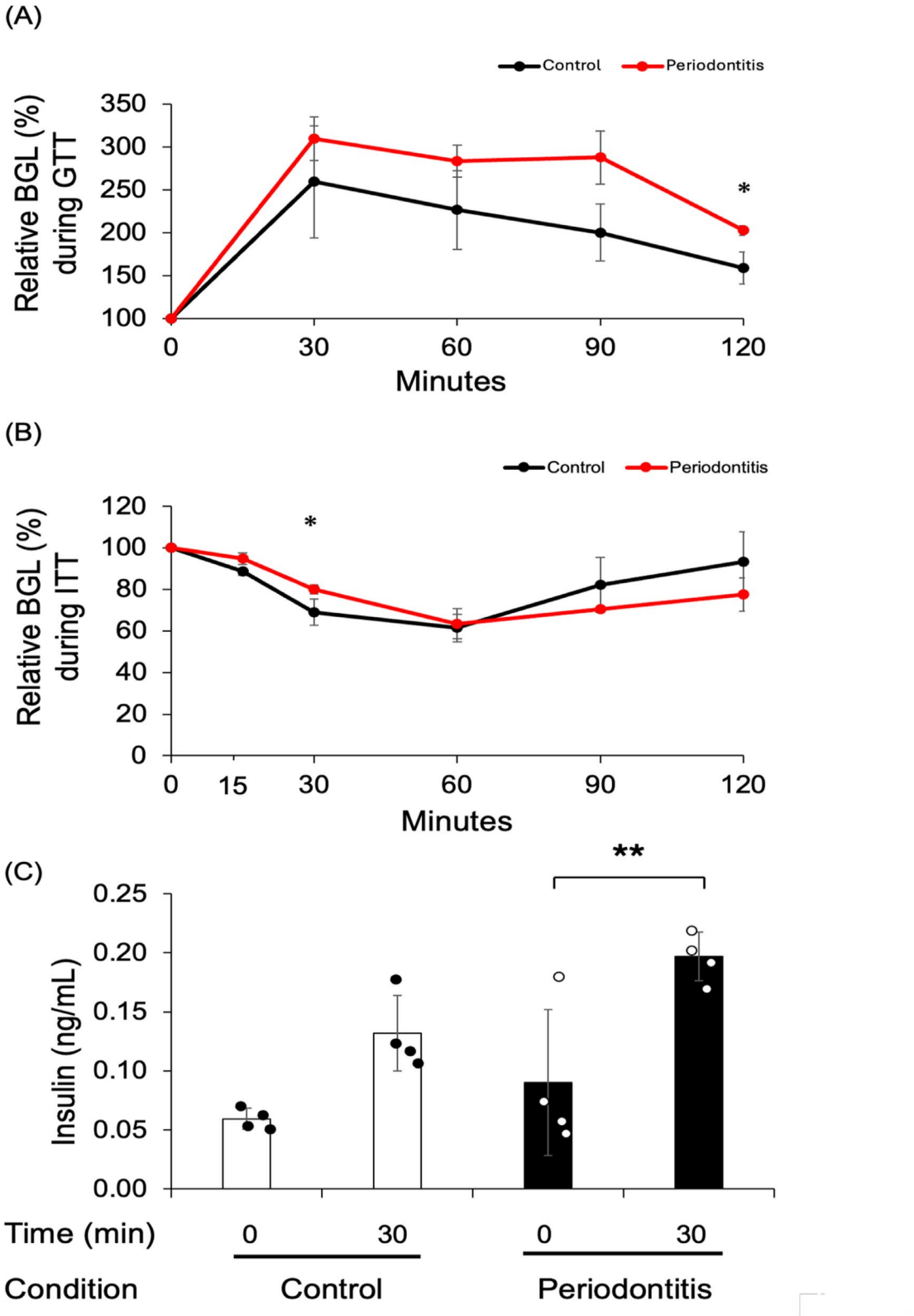


Fig. 5. Changes in blood glucose levels during tolerance tests and serum insulin concentration during insulin tolerance test. (**A**) Relative blood glucose levels in control and periodontitis groups during glucose tolerance test (GTT). Means and standard deviations are shown (control: n = 4, periodontitis: n = 4). (**B**) Relative blood glucose levels in control and periodontitis groups during insulin tolerance test (ITT). Means and standard deviations are shown (control: n = 4, periodontitis: n = 4). (**C**) Serum insulin concentrations before and 30 min after glucose administration for glucose tolerance test in control and periodontitis groups. Means and standard deviations are shown (control: n = 4, periodontitis: n = 4). BGL, blood glucose level. Statistical analysis was performed using the Mann–Whitney *U* test. *, $p < 0.05$; **, $p < 0.01$.

in the control group. Taxa that could not be classified at the genus or family level are denoted by the prefixes "g__", "f__", or "o__". *Coprococcus spp.*, a genus associated with insulin sensitivity, was significantly reduced in the periodontitis group (control, n = 4; periodontitis, n = 4, $p = 0.0302$; Fig. 6D). In contrast, *Lactobacillus spp*., which are linked to insulin resistance, were significantly increased ($p = 0.0075$; Fig. 6D), and *Prevotella spp*. were significantly increased ($p = 0.0417$; Fig. 6D).

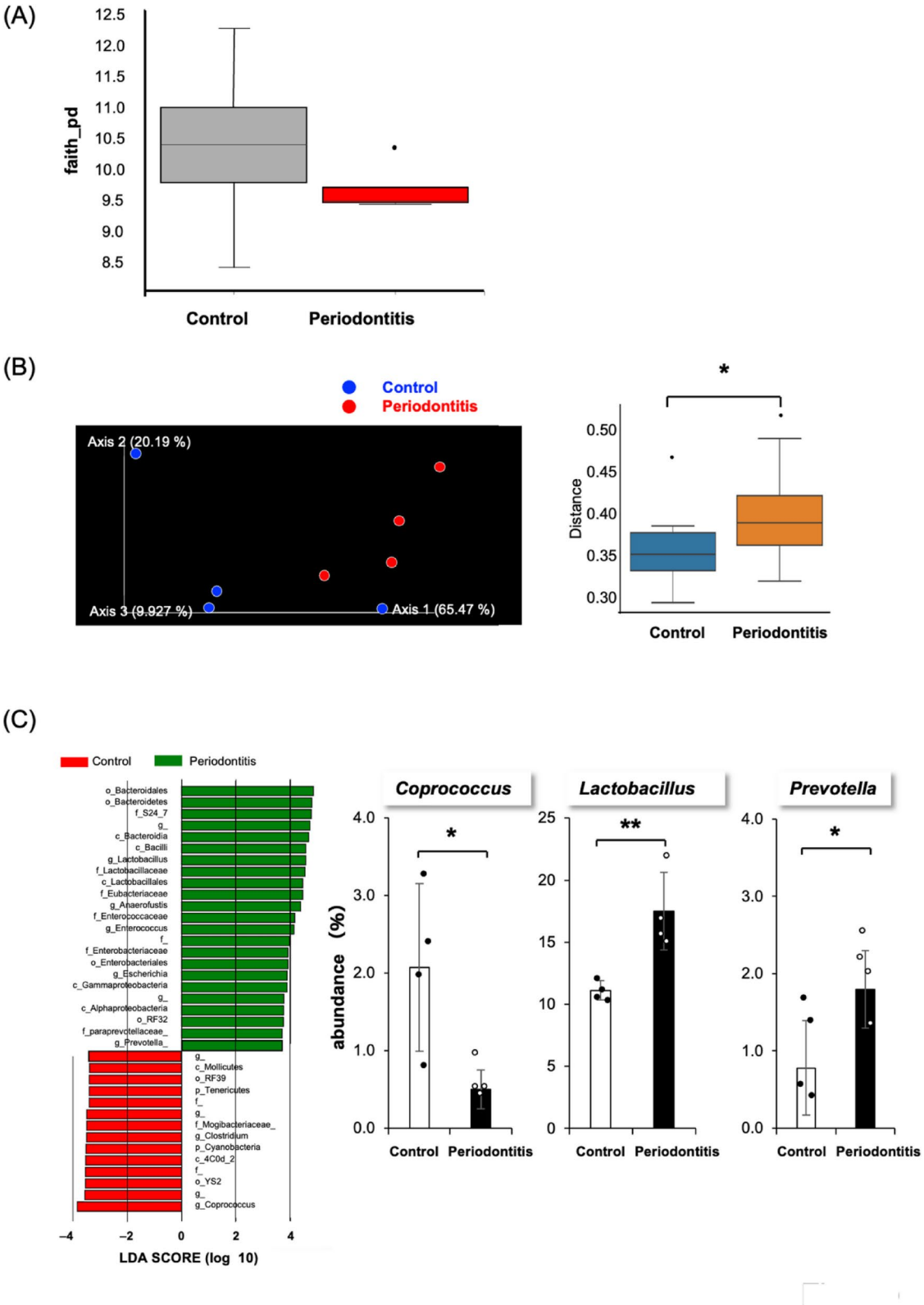
Discussion

In this study, we examined the effect of periodontal inflammation on diurnal blood glucose levels by employing a mouse ligature-induced periodontitis model with a CGM sensor attached. The findings suggest that the progression of periodontitis may disrupt glucose metabolism and the gut microbiota, potentially leading to or exacerbating insulin resistance, which could, in turn, affect the diurnal variation in blood glucose levels.

Previous studies on the relationship between periodontitis and DM have primarily utilized conventional glycemic control measures such as SMBG and HbA1c[13]. These indices are effective for assessing short- and long-term blood glucose levels. However, recent clinical research has highlighted that focusing on diurnal variations in blood glucose levels is crucial for managing DM and its complications[10,14]. Therefore, the use of CGM sensors, which allow for detailed monitoring of glucose variability throughout the day, is recommended. It is also beneficial for animal studies to concentrate on diurnal variations in glucose levels in basic research. However, to the best of our knowledge, no studies have been conducted using small animals, such as mice, to evaluate these fluctuations with CGM sensors. This is due to the relatively large size of commercially available CGM sensors[15], which are approximately 35 mm in diameter and 5 mm in thickness, rendering them unsuitable for use in mice. Additionally, the insertion of the sensor needle requires significant force to push it under the skin, creating a substantial burden on the animals. Moreover, CGM sensors designed for human use are typically inserted into the subcutaneous tissue of the upper arm, and their algorithms for measuring glucose levels in the interstitial fluid are optimized for that location. Therefore, it remains uncertain whether glucose concentrations in the subcutaneous tissues of mice correlate with actual blood glucose levels. Furthermore, continuous monitoring of the CGM sensor for 14 days, which is the typical usage period of the sensor, is expected to be challenging. To address these issues, we intraperitoneally anesthetized the mice and removed their fur using a hair removal cream. The back of each mouse was punctured with a 22G needle, and the CGM sensor needle was inserted and secured with tape. This method allows for the attachment of a CGM sensor (Fig. 7A). We performed SMBG on both control and periodontitis mice by comparing the simultaneous measurements obtained from CGM. In addition, we successfully measured the diurnal variation in blood glucose levels over 14 days and the maximum duration of the CGM sensor (Fig. 2). We measured the diurnal variation in glucose levels in both control and periodontitis mice immediately after inducing periodontitis. Our results showed that both groups remained in a hyperglycemic state until day 3 of CGM sensor application (Supplementary Fig. 1A, 1B). This hyperglycemia could be attributed to the metabolism of the anesthetics used during intraperitoneal anesthesia at the time of silk thread ligation and CGM sensor placement, consistent with the findings of a previous study[16]. On day 5 after sensor application, the diurnal variation in glucose levels stabilized in both the control and periodontitis groups, with no significant disturbances observed. However, after day 10, the diurnal variation in glucose levels was disrupted in the periodontitis group compared to the control group. The mean daily glucose level and TAR increased significantly (Fig. 3C). This disruption in blood glucose levels correlates with the progression of periodontitis, as measured by the degree of alveolar bone destruction. These findings suggest that inflammation in the periodontal tissues may influence blood glucose regulation.

At the beginning and during sample collection, there was no significant difference in body weight between the control and periodontitis groups (Fig. 1B). However, a notable decrease in food intake was observed in the periodontitis group after the 11th day post-periodontitis induction (Fig. 1C). This decline in food intake was thought to result from reduced feeding behavior caused by alveolar bone destruction and decreased masticatory function associated with the progression of periodontitis, consistent with previous studies[17]. On the other hand, both the GTT and ITT confirmed impaired glucose tolerance and insulin resistance in the periodontitis group (Fig. 5A, B). Furthermore, serum insulin concentrations before and 30 min after glucose administration in the GTT suggested potential hyperinsulinemia in the periodontitis group (Fig. 5C). Given that periodontal inflammation has been linked to an increased risk of glucose intolerance in the past[18], abnormal glucose metabolism related to periodontal inflammation may contribute to the rise in blood glucose levels in the periodontitis group, despite the decrease in caloric intake.

No significant difference in the serum levels of the pro-inflammatory cytokine TNF-α was observed between the control and periodontitis groups 14 days after induction (Fig. 4C). However, serum insulin and SAA levels were significantly elevated in the periodontitis group (Fig. 4A, B). Given that SAA, analogous to human CRP, was markedly increased in the periodontitis group, we hypothesized that the systemic effects of silk ligation-induced inflammatory alveolar bone destruction would be substantial. TNF-α has also been linked to the induction of insulin resistance[19,20]. In this study, periodontitis was induced solely by silk ligation. Still, previous reports indicate that serum TNF-α levels are elevated in models where periodontopathogenic bacteria, such as Pg, are introduced alongside silk ligation[21]. Therefore, future studies should examine diurnal variations in glucose levels in models where periodontopathogenic bacterial infection is combined with silk ligation. Additionally, no differences were observed between the two groups (Supplementary Fig. 2) in the histology of the liver and kidneys. Previous studies have reported that periodontal inflammation can affect the kidneys and liver[22,23]. It has been reported that impaired function of either the liver or the kidneys can lead to reduced gluconeogenic capacity, altered insulin dynamics, and increased glycemic variability, thereby affecting glucose metabolism[24,25]. However, given that the mice used in this study were healthy and were observed for only 14 days after silk ligation, we do not believe that periodontal inflammation had any adverse effects on these organs. Therefore, it is



considered highly probable that periodontal inflammation contributes to the diurnal variation in blood glucose levels demonstrated in this study.

Recent studies have reported that the composition of gut microbiota is altered in patients with type 2 DM, showing a decrease in alpha-diversity and an increase in beta-diversity[26,27]. In this study, the fecal microbiome analysis of periodontitis mice revealed that the gut microbiota diversity in the periodontitis group resembled that of patients with type 2 DM (Fig. 6A)[28]. Furthermore, this study demonstrated that inducing periodontitis in control mice led to a gut microbiota diversity comparable to that observed in patients with type 2 DM (Fig. 6B). Specifically, *Prevotella spp.*, which is linked to insulin resistance, was elevated in the periodontitis group, while

◀**Fig. 6**. Microbiome analysis. (**A**) The alpha-diversity between control and periodontitis groups. (**B**) The beta-diversity between control and periodontitis groups. (**C**) LEfSe-LDA score between control and periodontitis groups. Bacterial species dominant in each group are shown. Bacteria related to insulin sensitivity and resistance are compared between two groups. Control, n = 4; Periodontitis, n = 4. The relative abundances of the gut microbiota were compared using the Mann–Whitney *U* test, and the Faith's PD index was used to assess alpha-diversity. Beta-diversity was assessed via Principal Coordinate Analysis (PCoA) based on Bray–Curtis dissimilarities. To identify taxa showing significant intergroup differences, linear discriminant analysis effect size (LEfSe) was applied using the Galaxy platform (https://huttenhower.sph.harvard.edu/galaxy/), with a significance threshold of 0.05 and an LDA score cutoff of 2.0. Statistical differences in community composition were evaluated using PERMANOVA (permutational multivariate analysis of variance) with 999 permutations.

Coprococcus spp., associated with insulin sensitivity[29,30], was reduced (Fig. 6C). *Prevotella spp*. are commonly linked with carbohydrate-centered diets[31], and the influence of the environment on the onset and progression of DM has garnered increasing recognition. Previous studies have indicated that individuals with higher levels of *Coprococcus spp*. often exhibit greater insulin sensitivity, suggesting that a reduction in *Coprococcus spp*. may correlate with decreased insulin sensitivity and an elevated risk of type 2 DM[32]. The findings of this study imply that periodontitis may negatively impact glucose metabolism by altering gut microbiota, providing insights into a potential new mechanism through which periodontitis could contribute to the pathogenesis and progression of DM. However, this study did not investigate how treatment of periodontitis alters intestinal microbiota, and it is crucial to examine the effects of changes in intestinal microbiota on sugar metabolism in detail in future studies.

The results of this study suggest that the deterioration of oral health associated with the progression of periodontitis may influence not only systemic inflammation but also the gut microbiota, contributing to the onset and exacerbating of insulin resistance, which in turn may affect the diurnal variation in blood glucose levels. The limitation of this study is that the CGM sensor could only measure glucose levels for up to two weeks, making it difficult to observe diurnal variations over longer periods. It is hoped that future developments will lead to sensors capable of monitoring blood glucose variability for longer than two weeks. Additionally, this study confirmed that periodontitis induced solely by silk thread ligation in control mice disrupted the diurnal variation in blood glucose levels. Since it has been reported previously that Pg infection is associated with insulin resistance[31], future studies should investigate the effect of bacterial infection on blood glucose regulation. Moreover, it is crucial to examine how periodontitis influences diurnal variations in blood glucose levels in a mouse model of DM. Given that the exacerbating of periodontal inflammation associated with the progression of periodontal disease and the disruption of the oral gut microbiota are believed to contribute to insulin resistance—a key pathological condition of DM—periodontal therapy aimed at controlling periodontal tissue inflammation and oral bacterial infections is essential to prevent further deterioration in the health of patients with DM. Among patients with type 2 DM, periodontal disease has been reported to be associated with the number of missing teeth related to periodontal disease in end-stage renal failure diabetic patients undergoing hemodialysis[32]. Therefore, future clinical studies are needed to investigate the impact of periodontitis on the diurnal variation in blood glucose levels in patients with moderate to severe chronic periodontitis and to further explore the effects of periodontitis on glucose metabolism. This will help clarify the relationship between periodontitis and abnormal glucose metabolism, offering new insights into the prevention and management of DM and its complications.

In conclusion, periodontal inflammation disrupts the diurnal variation in blood glucose levels by using a ligature-induced periodontitis mouse model with a CGM sensor. This disruption appears to be associated with systemic inflammation and gut microbiota changes, potentially leading to insulin resistance. These findings highlight the significance of evaluating diurnal glucose variation as a novel aspect of the systemic impact of periodontal inflammation.

Methods

Establishment of the mouse periodontitis model by silk ligature and attachment of CGM sensors

To develop a mouse model of periodontitis, 9-week-old C57BL/6J wild-type male mice (CLEA Japan, Tokyo, Japan) were used following a partially modified version of the method described by Abe et al.[33]. After inducing general anesthesia via an intraperitoneal injection of 100 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride diluted in PBS, 5–0 silk threads were ligated around the neck of the maxillary bilateral second molar teeth to induce periodontal inflammation.

For the periodontitis mice described above, the fur on their backs was shaved, and a CGM sensor (FreeStyle Libre Pro, Abbott, Abbott Park, IL, USA) was attached and secured using adhesive tape (Fig. 7A). The CGM sensor employs an algorithm designed to estimate blood glucose levels based on the glucose concentrations in the interstitial fluid of the human upper arm. However, it was unclear whether the measurements obtained from the CGM sensor accurately reflected the actual blood glucose levels. To address this issue, SMBG was performed once daily using blood samples collected from the tail vein. The actual blood glucose values were compared with readings obtained from the CGM sensor. The control group underwent the same procedure with the CGM sensors attached to their backs. Mice that died during the experimental period, or whose CGM sensors were detached before the 10th day were excluded from the final analysis (Fig. 7B).

Mice were housed under specific pathogen-free (SPF) conditions with a 12-h light/dark cycle, a temperature of 22 ± 2 °C, and humidity maintained at 50 ± 10%. To minimize external influences on the gut microbiota, each

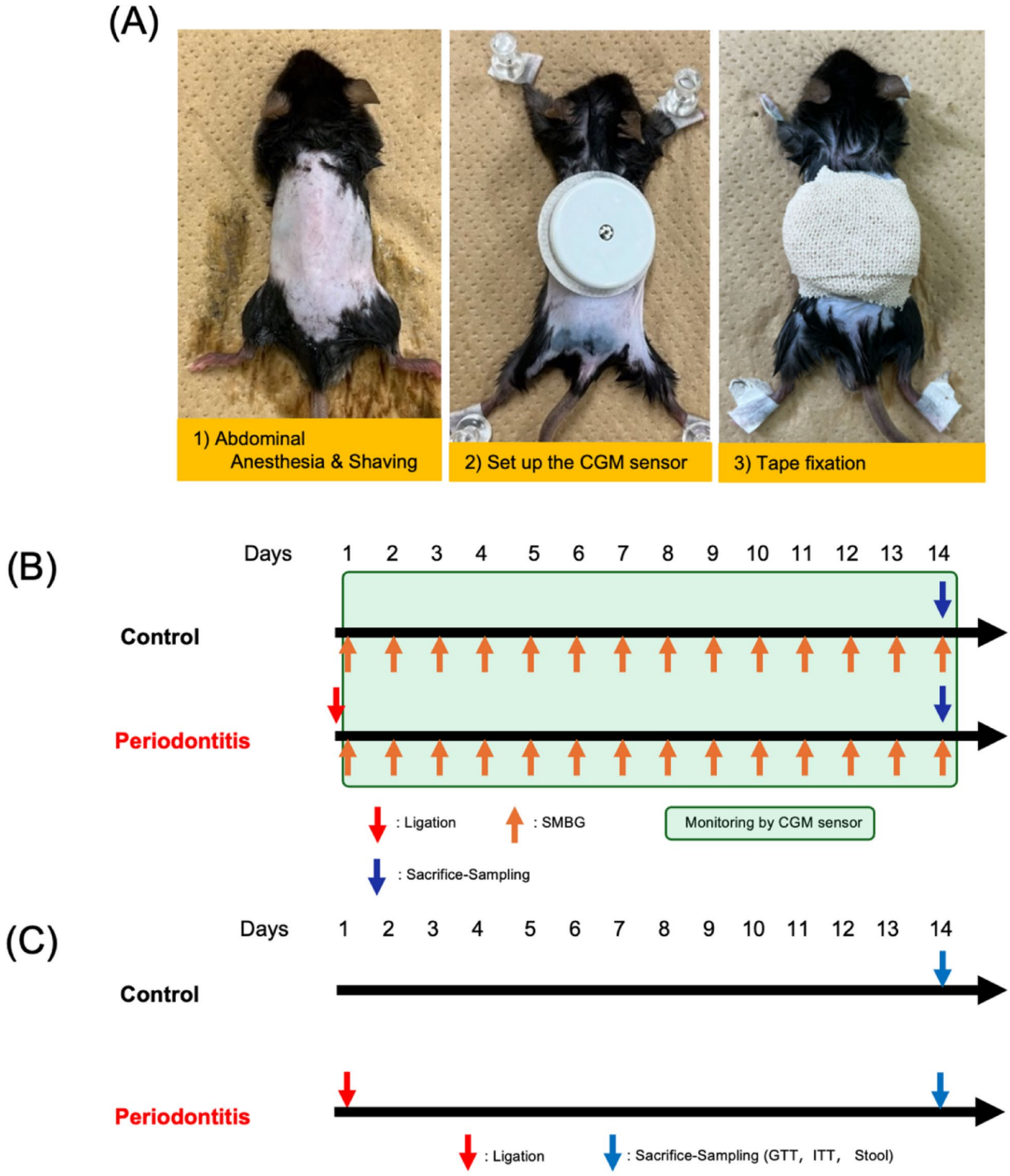


Fig. 7. Timeline and Methods for CGM Sensor Installation and Animal Experiments. (**A**) After dehairing the mouse's back with hair removal cream, the skin was pinched with tweezers and punctured with a 22G needle. The CGM sensor was then attached and secured using an adhesive tape. (**B**) Effect of periodontitis on the diurnal variation of blood glucose levels. A 5–0 silk thread was ligated around the cervix of the bilateral maxillary second molars in 9-week-old C57BL/6J wild-type male mice to induce periodontitis. Following the induction of periodontitis, SMBG was performed once daily from the tail vein of the mice to compare SMBG values with CGM measurements taken at the same time. Fourteen days post induction, the mice were euthanized, and samples of the jawbone, blood, kidney, liver, and feces were collected for analysis. (**C**) Effect of periodontitis on glucose metabolism. The glucose tolerance test (GTT) and insulin tolerance test (ITT) were conducted 14 days after periodontitis induction. Fasting periods for the tests were 16 h for the GTT and 4 h for the ITT. Insulin concentrations were measured before and 30 min after glucose administration during the GTT.

mouse was housed individually in a separate cage. Two weeks after periodontitis induction, periodontitis and control groups (healthy mice) were euthanized simultaneously using carbon dioxide gas. After euthanasia, the serum, maxillary bone, kidney, and liver samples were collected for analysis. All the animal experiments adhered to the ARRIVE guidelines and were approved by the Okayama University Animal Experimentation Committee (OKU-2023691).

For the CGM analysis using periodontitis-induced mice, the significance level was set at $\alpha = 0.05$, and the statistical power at $1 - \beta = 0.8$ ($\beta = 0.2$). Based on the preliminary data for TAR, assuming a mean difference of 37.5 between the two groups (control group: 21.5; periodontitis group: 59.0), a pooled standard deviation of 28.5 (control: 19.2; periodontitis: 37.8), and an equal allocation ratio (1:1), the required sample size was estimated to be $n = 11$ per group. To account for potential dropouts and technical issues, such as suture loss or CGM sensor detachment, the final sample size was increased to 14 animals per group. Sample size calculation was performed using an online tool developed by Nagashima K (A sample size determination tool for the two-sample t-test [Internet]. 2013 Jun 17; Available from: https://nshi.jp/contents/js/twomean12/ [in Japanese]).

A total of 28 mice (14 per group) were initially enrolled in this study. However, due to early detachment of the CGM sensors, four mice (one from the control group and three from the periodontitis group) were excluded from the analysis. As a result, CGM data were analyzed from 13 mice in the control group and 11 mice in the periodontitis group.

Confirmation of alveolar bone resorption due to periodontitis

Alveolar bone resorption was analyzed using a modified version of the method described by Abe et al.[30]. Bone specimens were prepared by autoclaving the mouse heads at 121 °C and 2 atm for 20 min (LSX-500, Tommy Seiko, Tokyo, Japan) to remove soft tissues. The remaining soft tissues were bleached with 30% hydrogen peroxide (Nacalai Tesque, Kyoto, Japan), neutralized using a sodium hypochlorite solution (Nacalai Tesque), and stained with eosin (Muto Chemical Co., Ltd., Tokyo, Japan) for 5 min and methylene blue (Merck KGaA, Darmstadt, Germany) for 10 sec. The specimens were then dried thoroughly. The alveolar bone resorption was measured using a stereomicroscope (SZ-LW61 T2; Olympus, Tokyo, Japan). Measurements were performed at six points.

- The centro-lingual fissure and centro-lingual cusp of the first molar
- Proximal palatal cusp, palatal fissure, and centro-lingual cusp of the second molar
- The palatal cusp of the third molar

The vertical distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was recorded at each point. The average vertical distance between teeth was calculated to determine the degree of bone resorption. One sample was collected per jaw, and specimens with missing teeth during preparation were excluded from the analysis. The evaluation of alveolar bone resorption was performed by MKT and CNK (dentists with experience in periodontal tissue analysis). The alveolar bone was measured by MKT and the measurements were verified by CKN. All measurements were conducted in a blinded manner, without knowledge of the group assignments, and the evaluation criteria were applied consistently. Visual comparison across bilateral and multiple measurement sites was used throughout the process to ensure consistency.

Comparison and analysis of CGM and SMBG measurements

Following the induction of periodontitis, blood samples were collected once daily from the tail vein of the mice, and blood glucose levels were measured using a self-measured blood glucose meter (FreeStyle Precision Glucose Measurement Electrode, Abbott; Fig. 1B). CGM sensors were linked to a CGM Reader (FreeStyle Libre Reader, Abbott) through near-field communication (NFC), and the collected data were analyzed using FreeStyle Libre software (Windows version, Abbott). The SMBG readings were directly compared with the CGM measurements obtained simultaneously. Additionally, average daily glucose levels and the percentage of daily hyperglycemia were analyzed for both the control and periodontitis groups using CGM data. To validate the accuracy of CGM measurements, SMBG was performed immediately after each CGM scan in real time. The time difference between CGM readings and SMBG measurements was standardized to within 5 min, and only paired data within this interval were used for analysis. CGM sensors were scanned once daily between 6:00 and 8:00 PM. At the same time, tail vein blood samples were collected to measure blood glucose levels using SMBG. The reliability of CGM readings in mice was evaluated by comparing CGM-estimated glucose values with SMBG measurements obtained at the same time points.

Measurement of body weight and food intake

Body weight was measured at the start of the experiment and 14 days after periodontitis induction for comparison. Mice were provided with 7 g of standard pellets (Oriental Yeast Co., Ltd., Tokyo, Japan) per day according to their dietary requirements. Daily food intake was recorded by measuring the weight of the remaining food.

Enzyme-linked immunosorbent assays (ELISA)

Fresh blood was collected from the hearts of both the control and periodontitis groups. The blood was then centrifuged at $875.17 \times g$ for 10 min at 4 °C, and the serum was separated. Serum insulin concentrations were measured using the Mouse/Rat Insulin Assay Kit (Morinaga Institute of Biochemistry Inc., Yokohama, Japan). Serum amyloid A (SAA) concentration was determined using the Mouse SAA ELISA Kit (Tridelta Development Ltd., Kildare, Ireland). SAA corresponds to C-reactive protein (CRP), a systemic marker of human inflammation. Serum tumor necrosis factor (TNF)-α was quantified using the Mouse TNF-α ELISA Kit (BioLegend, San Diego, CA, USA). The absorbance was measured at 450/630 nm using an iMark Microplate Reader and Microplate

Manager Software 6.3 (both Bio-Rad, Hercules, CA, USA). Each ELISA measurement was performed following the manufacturer's instructions.

Glucose and insulin tolerance tests

Both glucose tolerance test (GTT) and insulin tolerance test (ITT) were conducted 14 days after periodontitis induction (Fig. 7C). GTT and ITT analyses were conducted on a separate cohort of newly added mice (n = 4 per group), independently of the CGM experiments. For the GTT, glucose diluted in saline (0.1 mL/10 g body weight) was administered intraperitoneally to mice after a 16-h fast. Blood samples were collected from the tail vein of mice at 0, 30, 60, 90, and 120 min to measure blood glucose levels. For the ITT, insulin (Fujifilm Wako Pure Chemicals, Osaka, Japan) dissolved in 0.01 M dilute hydrochloric acid was administered intraperitoneally at a dose of 0.75 units/kg after a 4-h fast. Blood samples were collected from the tail vein at 0, 15, 30, 60, 90, and 120 min to post-administration to measure blood glucose levels. Additionally, for the GTT, serum insulin concentrations in the mice were measured at 0 and 30 min after administration using the ELISA technique described before.

Histological analysis of liver and kidney

Given that periodontitis induces systemic inflammation and is implicated in the development of insulin resistance and metabolic dysfunction, histopathological alterations in the liver and kidney—key organs involved in glucose regulation and waste elimination were assessed. Portions of the kidneys and livers from both control and periodontitis mice were fixed in 4% paraformaldehyde solution (pH 7.4; Fujifilm Wako Pure Chemicals, Osaka, Japan) for 24 h. After fixation, tissues were dehydrated using a graded ethanol series and embedded in paraffin to form blocks. These blocks were sectioned into 5 µm-thick slices to create paraffin sections. The paraffin sections were deparaffinized with xylene (Nacalai Tesque, Kyoto, Japan) and rehydrated by immersing the slides in an ethanol series, starting from anhydrous to 70% ethanol. Hematoxylin and eosin (H-E) staining was performed, followed by dehydration via sequential immersion in 70% ethanol to anhydrous ethanol, followed by immersion in xylene. The sections were mounted with Mount-Quick (Dido Sangyo Co., Ltd., Tokyo, Japan) and covered with coverslips. Once the slides were dried, the tissue samples were examined under an optical microscope (BX-50, Olympus, Tokyo, Japan) to observe the histological features.

Microbiome analysis

Mouse feces were collected from mice prior to the administration of the GTT and ITT for microbiome analysis (n = 4 each group). This approach was adopted to investigate the composition and diversity of gut microbiota and their potential correlation with metabolic parameters.

Mouse feces were collected 14 days after periodontitis induction and stored at −30 °C. DNA extraction from fecal samples and microbiome analysis via 16S rRNA sequencing were outsourced to the Biotechnology Research Institute (Kyoto, Japan). Sequencing targeted the V3–V4 region of the 16S rRNA gene using universal primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′). Sequencing data obtained using next-generation sequencing (Illumina MiSeq) were assessed for library density and quality control by Biotechnologies Inc. The sequencing data were analyzed using the QIIME2 software (https://qiime2.org/) based on the information provided by the Biotechnology Research Institute. Alpha-diversity (Faith's PD), beta-diversity (Unweighted UniFrac distance), and group analyses (LEfSe-LDA scores) were performed for each sample.

Statistical analysis

Comparisons between the two groups of longitudinal data were conducted using a mixed-effects model (REML) with Bonferroni's multiple comparison test. The Shapiro–Wilk test was used to assess normality, and the Mann–Whitney *U* test was applied for non-normally distributed data between groups. Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA).

The relative abundances of the gut microbiota and the Faith's PD index for alpha-diversity were compared using the Mann–Whitney *U* test, which is appropriate for sparse and non-normally distributed data.

Beta-diversity was assessed via Principal Coordinate Analysis (PCoA) based on Bray–Curtis dissimilarities. To evaluate differences in overall microbial community structure, we performed permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis dissimilarities. In addition, the homogeneity of multivariate dispersions among groups was assessed using the analysis of multivariate homogeneity of group dispersions (PERMDISP). The analysis showed no significant differences in dispersion ($F = 0.145$, $p = 0.717$), confirming that the assumption of homogeneity was satisfied for the PERMANOVA.

To identify taxa showing significant intergroup differences, linear discriminant analysis effect size (LEfSe) was applied using the Galaxy platform (https://huttenhower.sph.harvard.edu/galaxy/), with a significance threshold of 0.05 and an LDA score cutoff of 2.0.

A p value < 0.05 was considered statistically significant.

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request. The metagenome raw sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under the accession number [PRJNA1277107].

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Author contributions

MKT and K. Omori contributed to the conception, design, data acquisition, analysis, and interpretation of the data and drafted and critically revised the manuscript. CKN, FK, and TI acquired, analyzed, and interpreted the data. MN and KG performed the microbiome analysis. KH, YSI, K. Okubo, SN, AI and TS interpreted the data. JW contributed to the conception, data analysis, and interpretation and critically revised the manuscript. ST contributed to the conception, design, data analysis, and interpretation and critically revised the manuscript. All authors have approved the final manuscript and agreed to be accountable for all aspects of this study.

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Declarations

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to K.O.

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