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Safety profile and antimicrobial potential of a phosphorylated pullulan cetylpyridinium chloride complex assessed using in vitro and in vivo models

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Cetylpyridinium chloride (CPC) is widely used as an oral antiseptic, but its effectiveness is limited by rapid clearance from oral surfaces. Phosphorylated pullulan (PP), a biocompatible polysaccharide derivative, enhances CPC retention on calcium-rich enamel and forms a PP–CPC complex designed for sustained antimicrobial activity. This study evaluated the preclinical safety of PP–CPC using integrated in vitro and in vivo models. Cytotoxicity was assessed in three human-derived cell types. Systemic toxicity was examined by single- and repeated-dose oral administration in rats and mice, and local mucosal irritation was evaluated in a hamster buccal-pouch model. Exploratory analyses of oral microbiota and fecal microflora were also performed. PP alone exhibited no detectable cytotoxicity, whereas CPC and PP–CPC at higher concentrations induced epithelial cell shrinkage and reduced metabolic activity. No mortality, treatment-related behavioral abnormalities, or clinically relevant systemic toxicity was observed in animal studies. Renal tubular epithelial hypertrophy was observed in some CPC-containing groups; however, no detectable functional renal impairment was identified by serum biochemical analysis during the study period. PP–CPC did not induce biologically meaningful buccal mucosal irritation. Exploratory oral microbiota analysis showed stable total bacterial abundance, while T-RFLP profiles showed time-dependent tendencies in community patterns in CPC-containing groups. These findings support further development of PP–CPC as a candidate oral material and provide a basis for carefully monitored early-phase clinical evaluation.

Keywords Cetylpyridinium chloride, Phosphorylated pullulan, Oral antiseptic, Preclinical evaluation, Oral microbiota

Oral biofilm-related diseases, including dental caries and periodontitis, are major global health challenges[1–3]. As populations age and an increasing number of individuals retain their natural dentition[4,5], preventive strategies aimed at effective biofilm control are becoming increasingly important[6,7]. Although conventional mouthwashes containing antiseptic agents provide transient bacterial suppression, their limited persistence highlights the need for formulations with prolonged activity and well-established safety profiles.

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Chlorhexidine has long been regarded as the gold standard oral antiseptic in several countries because of its potent and sustained antimicrobial effects[8,9]. However, its routine use is often restricted owing to well-documented adverse effects, including tooth staining, taste disturbance, and mucosal irritation[10–12], as well as regulatory limitations that vary across regions. In Japan, these considerations have led to a preference for alternative antiseptic agents with favorable tolerability profiles for daily oral care.

Cetylpyridinium chloride (CPC) is a cationic antiseptic widely used in oral care products, such as mouthwashes, toothpastes, and lozenges[13–15], particularly in regions where chlorhexidine use is limited. Although CPC exhibits broad-spectrum antimicrobial activity, it is rapidly cleared from oral surfaces, resulting in limited residual effects. Accordingly, strategies enhancing CPC retention may substantially improve its clinical utility.

Pullulan is a naturally derived polysaccharide produced by *Aureobasidium pullulans* and is commonly used as a food additive and pharmaceutical excipient with an established safety record. We previously developed a material with structural features resembling those of glass ionomer cement using phosphorylated pullulan (PP), which enabled efficient binding to calcium ions[16–18]. This suggests that PP can function as a carrier capable of anchoring CPC to calcium-rich tooth surfaces, thereby prolonging its local antibacterial action.

Preliminary studies demonstrated that the PP–CPC complex exhibited sustained antiseptic activity on artificial tooth surfaces, even at low concentrations[18]. However, the cytotoxicity and systemic safety of this complex have not been fully characterized, and its tolerability under conditions relevant to oral administration remains unclear. Because both PP and CPC are intended for human use, a comprehensive safety evaluation across multiple experimental models is essential before clinical translation.

Whereas our previous study established the retention, sustained-release, and antimicrobial properties of PP–CPC on hydroxyapatite surfaces[18], the present study focused on its preclinical local and systemic safety using complementary in vitro and in vivo models. Cytotoxicity was evaluated in human-derived cells, followed by single- and repeated-dose oral toxicity studies in rats and mice and buccal-pouch mucosal irritation testing in hamsters, including GLP-compliant studies. The aim was to characterize the preclinical safety profile of PP–CPC and provide a foundation for its further development as a candidate oral care material.

Results

In vitro cytotoxicity

To evaluate in vitro cytotoxicity, three human cell types (HeLa, KB, and human gingival fibroblasts; HGFs) were exposed to various concentrations of PP, CPC, PP–CPC, or phosphate-buffered saline (PBS) for 3 min. Twenty-four hours after exposure, cellular morphology was examined by phase-contrast microscopy, whereas cell number and viability were determined by immunofluorescence and MTT assay, respectively.

No morphological changes were observed in the cells exposed to PP or PBS at any of the tested concentrations. In contrast, exposure to PP–CPC and CPC solutions induced marked cell shrinkage at both tested concentrations (Supplementary Fig. S1A). When HeLa cells were exposed to serial dilutions ranging from 0.0001 to 0.1%, no morphological alterations were observed in PP-treated cells at any concentration, whereas cell shrinkage was evident in cells treated with PP–CPC and CPC at concentrations ≥ 0.01% (Supplementary Fig. S1B).

When three human cell types (HeLa, KB, and HGFs) were exposed to serially diluted solutions, the cell counts remained largely unchanged at all concentrations of PP. Similarly, the cell counts remained unaffected at concentrations of 0.0001% and 0.001% for either PP–CPC or CPC. At 0.01%, both PP–CPC and CPC induced a significant reduction in the cell count, whereas an apparent increase in the cell count was observed at 0.1%. These trends were consistent across all three cell types (Fig. 1A). In contrast, cell viability measured by the MTT assay started to decline at concentrations as low as 0.001% for both PP–CPC and CPC, dropping to about 20% of the control level at 0.01% or higher (Fig. 1B).

Rat toxicity study

To assess repeated oral exposure, rats received ultrapure water, PP, CPC, or PP–CPC as the sole drinking fluid for 28 days. The study was conducted in two sequential experimental phases. In the first phase, clinical condition, body weight, food and water intake, blood biochemical parameters, and histopathology were evaluated. Approximately 2 months later, the second phase was conducted to examine total bacterial abundance and oral bacterial community profiles longitudinally.

All rats in the first experimental phase (three males and three females per treatment group) exhibited normal behavior and body weight gain throughout the 28-day administration period (Fig. 2A). Food and water intake showed no apparent treatment-related change (Fig. 2B and C). Body weight increased over time in both males and females, with no significant differences among groups within either sex.

Blood biochemical analyses, based on four animals of each sex per treatment group from the two experimental phases, revealed occasional minor variations, including slight changes in alanine aminotransferase or creatinine levels in some CPC-exposed animals; however, values remained within physiological ranges and were not associated with clinical abnormalities (Supplementary Table S1).

Histopathological examination of the prespecified subset of eight rats identified several renal findings in individual female rats in the CPC and PP–CPC groups, including inflammatory cell infiltration in the renal pelvic submucosa, dilatation and calcification of the outer medullary tubules, regeneration of cortical tubules, and thickening of the renal pelvic mucosa (Supplementary Table S2). Hepatic findings were observed in both control and treated animals, whereas no abnormal findings were detected in the spleen or gingival tissues.

In the second experimental phase, longitudinal analysis of the oral microbiota in two males and two females per treatment group showed no marked temporal change in relative total bacterial abundance, with no significant differences among the treatment groups at any observation time point (Fig. 3A). At baseline, T-RFLP-based community profiles showed no apparent clustering by treatment group. At week 2, partial clustering was

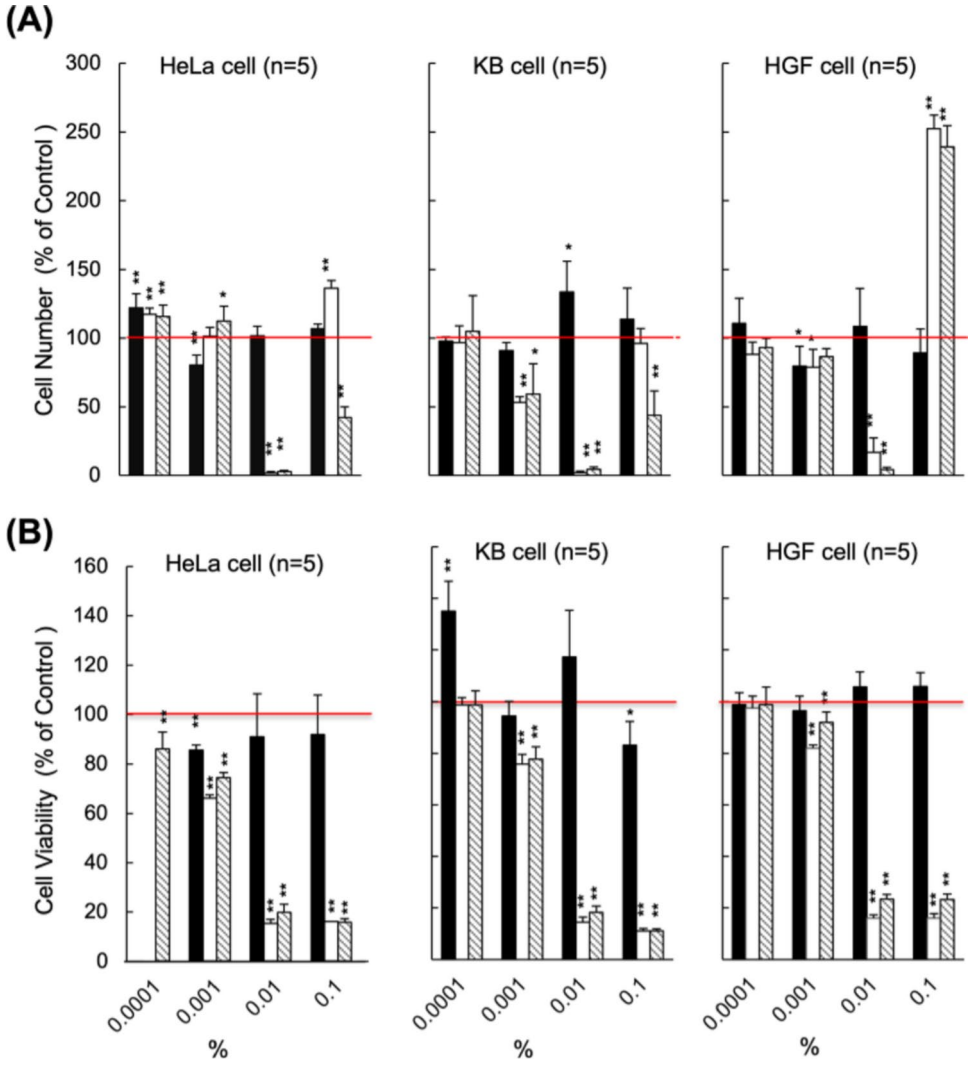


Fig. 1. In vitro effects of PP and PP–CPC solutions on the cellular activity of human-derived cells. HeLa, KB, and human gingival fibroblasts (HGFs) were exposed for 3 min to phosphorylated pullulan (PP), cetylpyridinium chloride (CPC), or PP–CPC at concentrations ranging from 0.0001% to 0.1%. For PP–CPC, each indicated concentration represents equal concentrations of PP and CPC; for example, 0.01% PP–CPC contained 0.01% PP and 0.01% CPC. Phosphate-buffered saline without calcium and magnesium [PBS(–)] served as the negative control. After exposure, the cells were rinsed with PBS(–) and cultured for 24 h. **(A)** Cell number determined by automated array scanning. **(B)** Cellular metabolic activity assessed using the MTT assay. Values are expressed relative to the mean value of the PBS(–) control, which was defined as 100%. Data are presented as the mean ± standard deviation from five independent experiments ($n = 5$). Statistical comparisons were performed between each treatment condition and the corresponding PBS(–) control using Student's *t*-test. *: $p < 0.05$; **: $p < 0.01$. Black bars, PP solution; white bars, CPC solution; shaded bars, PP–CPC solution.

observed among samples from the CPC and PP–CPC groups. At week 4, samples from the CPC-containing groups tended to cluster separately from those from the control and PP groups (Fig. 3B).

GLP-compliant mouse toxicity studies

To evaluate systemic toxicity under GLP-compliant conditions, two complementary mouse studies were conducted: a 14-day repeated-dose oral administration study and a single-dose oral administration study followed by a 14-day observation period.

Repeated-dose oral administration

In the repeated-dose study, mice received water for injection, 10% PP, or PP–CPC containing 0.1% PP and 0.1% CPC once daily by oral gavage for 14 days. Clinical condition, body weight, hematological and biochemical parameters, organ weights, histopathological findings, and fecal microflora were evaluated.

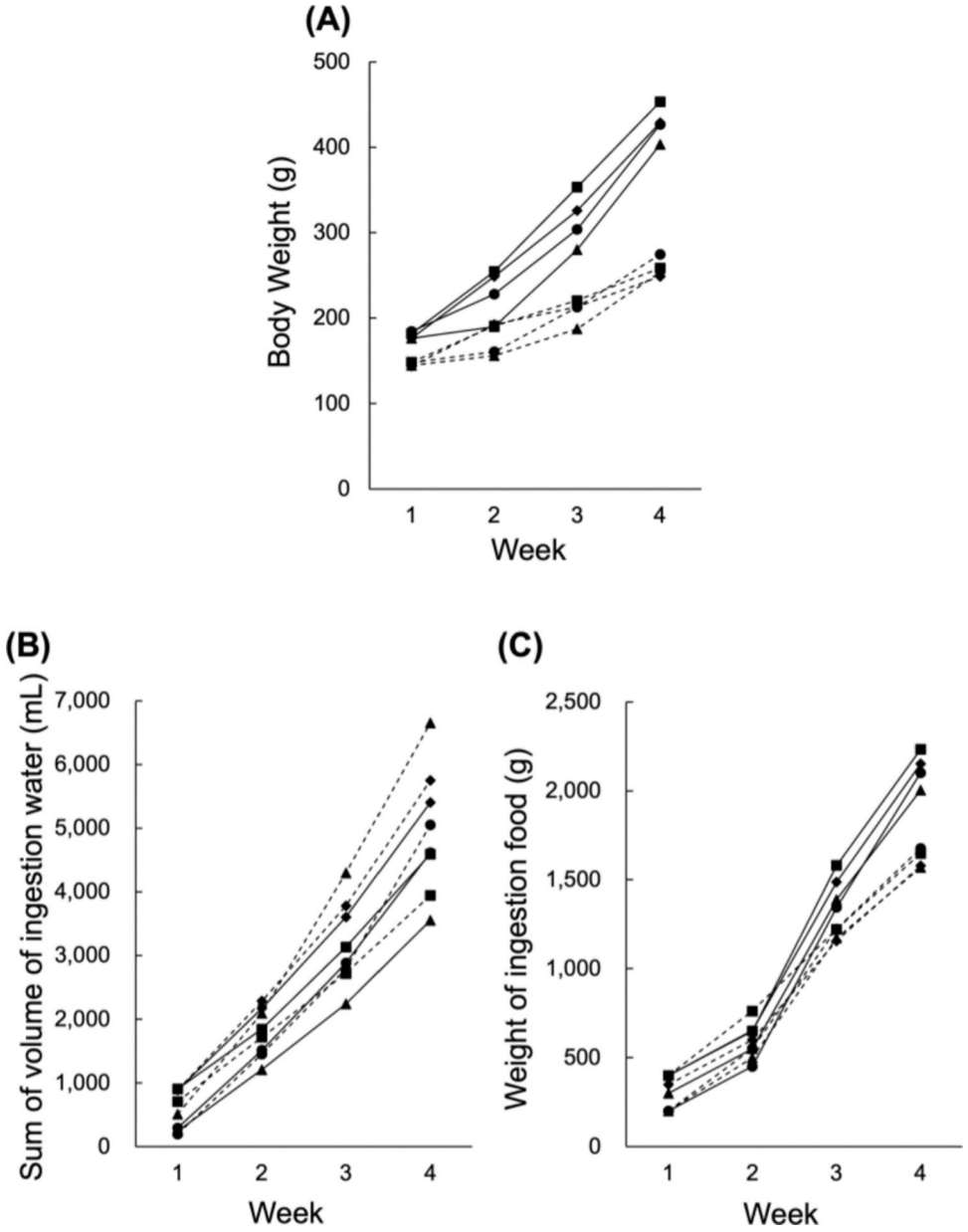


Fig. 2. In vivo effects of PP and PP–CPC on body weight and food and water intake in rats during 28-day oral administration. Male and female Sprague–Dawley rats received ultrapure water, 0.01% phosphorylated pullulan (PP), 0.01% cetylpyridinium chloride (CPC), or PP–CPC containing 0.01% PP and 0.01% CPC as the sole drinking fluid for 28 days. Animals were housed individually, allowing water and food intake to be monitored for each rat. (**A**) Changes in body weight during the administration period, (**B**) Total water intake, and (**C**) Total food intake over the 28-day period. Data are presented as the mean ± standard deviation ($n = 3$ per sex per group). Solid lines indicate males and dashed lines indicate females. Squares, ultrapure-water control; diamonds, PP; triangles, CPC; circles, PP–CPC.

No mortality or treatment-related behavioral abnormalities were observed. Body weight increased normally in all groups, with minor sex-dependent differences (Supplementary Fig. S2A). Statistically significant reductions in neutrophil counts were observed in females receiving PP–CPC (0.1% PP and 0.1% CPC), and reduced lymphocyte counts were observed in females receiving 10% PP; however, no corresponding abnormalities were detected in blood biochemical parameters (Supplementary Table S3).

Isolated organ findings were noted, including a single case of renal cysts in a male receiving PP (10%) and sporadic findings such as splenic hypertrophy, lung nodules, or ovarian cysts in individual females across different groups, including controls (Supplementary Table S4). Organ weight analysis revealed minor variations, including decreased brain weight in males treated with high-dose PP–CPC and increased epididymal weight in males treated with high-dose PP (Supplementary Fig. S3).

Histopathological examination in the repeated-dose mouse study identified epithelial hypertrophy of renal collecting tubules in all males and two females receiving PP–CPC (0.1% PP and 0.1% CPC), and distal tubular epithelial hypertrophy in two females in the same group. Other findings were sporadic and were observed across different groups, including controls. These findings are summarized as incidence data in Table 1.

In the exploratory fecal microflora analysis performed in male mice, cultivable fecal bacteria were detected using the applied culture-based method. No clear treatment-associated changes were observed among the detectable bacterial groups after repeated administration. *Bifidobacterium* and lecithinase-positive clostridia remained below detection limits throughout the study (data not shown).

Single-dose oral administration

In the single-dose study, mice received a single oral dose of water for injection, 20% PP, or PP–CPC containing 0.1% PP and 0.1% CPC and were observed for 14 days. Clinical signs, body weight, and gross necropsy findings were assessed.

Mice tolerated a single oral administration of PP (20%) and PP–CPC (0.1% PP and 0.1% CPC) without mortality or signs of systemic toxicity. Body weight changes remained within the normal range (Supplementary Fig. S2B). Gross necropsy revealed a single renal cyst in one animal in the PP-treated group (Supplementary Table S5).

Hamster mucosal irritation test

To evaluate local oral mucosal tolerability, male hamsters received once-daily topical application of 1% PP, 0.05% CPC, or PP–CPC containing 0.05% PP and 0.05% CPC to the right buccal pouch for 4 consecutive days; the untreated left buccal pouch served as an internal control. General condition and body weight were monitored, and macroscopic and histopathological mucosal findings were assessed approximately 24 h after the final application using predefined grading criteria.

All hamsters maintained normal activity and body weight throughout the study (Supplementary Fig. S2C). Macroscopic examination of the buccal-pouch mucosa showed no erythema, eschar formation, ulceration, or edema in any group. Histopathological evaluation showed no epithelial erosion, vascular congestion, or edema. Minimal leukocyte infiltration was observed in one CPC-treated animal, whereas no such finding was observed in the PP or PP–CPC groups. No findings of grade 2 or higher were observed in any group (Table 2).

Discussion

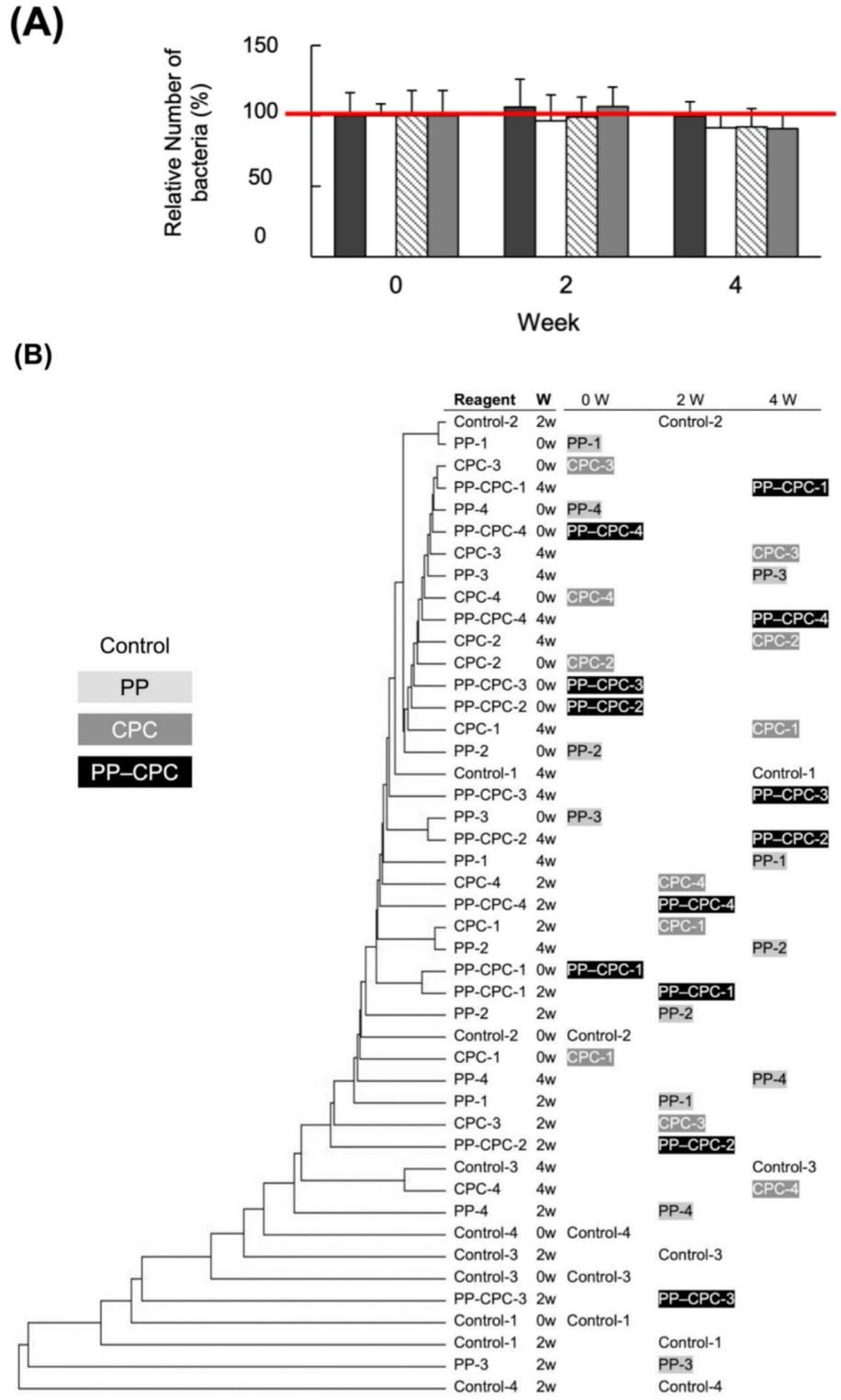
The present study provides an integrated preclinical safety assessment of PP–CPC using complementary in vitro and in vivo models. PP itself showed little detectable cytotoxicity, whereas CPC-containing formulations induced concentration-dependent changes in cellular morphology and metabolic activity. No overt systemic toxicity or biologically meaningful buccal mucosal irritation was detected under the tested conditions, although renal histopathological findings in the rat and repeated-dose mouse studies require cautious interpretation. Exploratory microbiota analyses showed no marked change in relative total bacterial abundance, but suggested time-dependent differences in community profiles.

These findings extend our previous work, which demonstrated that PP–CPC retained CPC on tooth surfaces, sustained CPC release, and suppressed oral biofilm formation[18]. Whereas the previous study focused primarily on retention and antimicrobial function, the present study addresses the local and systemic safety questions relevant to further clinical development.

The in vitro findings indicate that PP itself had little detectable cytotoxicity under the experimental conditions, whereas CPC-containing formulations altered epithelial cell morphology and metabolic activity at higher concentrations. This pattern aligns with the known properties of CPC as a cationic quaternary ammonium compound that interacts with negatively charged cellular and microbial membranes[19]. The reduction in MTT activity observed in the CPC and PP–CPC groups, even when marked decreases in cell number were not apparent, may reflect early suppression of cellular metabolic activity or mitochondrial function before overt cell detachment or loss of viability[20–22]. Therefore, the in vitro results should be interpreted as evidence that the cellular response was primarily driven by CPC exposure, while PP did not appear to add substantial cytotoxicity.

The concentrations and exposure periods in this study were selected to encompass both clinically relevant and conservative exposure conditions for a candidate oral material. CPC concentrations ranging from 0.01% to 0.1% were used across the experiments to represent anticipated local-use concentrations and higher conservative exposure conditions[14,15]. The 0.05% CPC concentration used in the hamster study corresponds to the upper concentration specified in Japanese quasi-drug standards for medicated dentifrices containing CPC[23]. The PP–CPC concentration of 0.1% PP and 0.1% CPC used in the mouse studies represented a higher exposure condition specified in the approved toxicity study protocols. The 3-min in vitro exposure was intended to represent a short, direct mucosal or epithelial contact period and was comparable to short exposure models previously used to evaluate the cytotoxicity of oral rinses in gingival fibroblasts and epithelial cells[24], while longer in vivo exposure studies were designed to detect potential cumulative effects after repeated administration. Because PP–CPC is intended for local oral application rather than systemic administration, evaluation of both short-term epithelial contact and repeated oral exposure was necessary to estimate a reasonable safety margin before clinical translation.

The in vivo findings should be interpreted in light of the distinct purpose of each animal study. The GLP-compliant mouse and hamster studies were conducted at an external testing facility using individually identified animals and approved protocols, supporting the reliability of the safety assessments. The repeated-dose mouse study was designed to detect systemic toxicity after oral administration, and the microscopic findings were summarized as incidence data rather than the graded severity of scores. In contrast, the hamster buccal-pouch study directly assessed local mucosal tolerability using predefined macroscopic and histopathological grading



criteria, with findings limited to minimal leukocyte infiltration in one CPC-treated animal. This distinction is important for PP–CPC, which is intended for local oral application, because systemic organ toxicity and direct mucosal compatibility represent complementary but different aspects of its preclinical safety profile.

Renal findings differed between the rat and mouse studies. In the rat study, individual female rats in the CPC and PP–CPC groups showed inflammatory cell infiltration in the renal pelvic submucosa, dilatation and calcification of the outer medullary tubules, regeneration of cortical tubules, and thickening of the renal pelvic mucosa. Because histopathological evaluation was limited to one male and one female per treatment group, the relationship of these findings to treatment could not be determined. In the repeated-dose mouse study, collecting-tubule epithelial hypertrophy was observed in all males and two females receiving PP–CPC, and distal tubular epithelial hypertrophy was additionally observed in two females in the same group. Studies of the related quaternary ammonium compound benzalkonium chloride have shown that systemic distribution and

◀**Fig. 3**. Effects of repeated oral administration of PP and PP–CPC on the oral microbiota in rats. Male and female Sprague–Dawley rats received ultrapure water, 0.01% phosphorylated pullulan (PP), 0.01% cetylpyridinium chloride (CPC), or PP–CPC containing 0.01% PP and 0.01% CPC as the sole drinking fluid for 28 days. Oral bacterial samples were collected from the buccal surface of the left molar at baseline (week 0) and at weeks 2 and 4. The oral-microbiota cohort comprised two males and two females per treatment group ($n = 4$ per group). The same animals were sampled longitudinally at baseline and at weeks 2 and 4. (**A**) Relative total bacterial abundance determined by real-time PCR targeting the bacterial 16 S rRNA gene. Values represent the mean with error bars indicating standard deviation. Values at each time point are expressed relative to the corresponding baseline value at week 0, which was defined as 100%. Data are presented as the mean ± standard deviation. Black bars, ultrapure–water control; white bars, PP solution; shaded bars, CPC solution; gray bars, PP–CPC. (**B**) Hierarchical clustering of bacterial community profiles based on terminal restriction fragment length polymorphism (T-RFLP) analysis. The dendrogram was generated using MicrobiotaProfiler software to visualize similarities among samples collected at weeks 0, 2, and 4. Sample labels indicate treatment group, animal number, and sampling time. Animal numbers 1 and 2 represent males, and numbers 3 and 4 represent females. The samples CPC-2 at week 2, Control-2 at week 4, and Control-4 at week 4 were unavailable and were therefore excluded from the analysis. Baseline: $n = 4$ per group; week 2: $n = 4$ per group except the CPC group ($n = 3$); week 4: $n = 4$ per group except the control group ($n = 2$).

	Male			Female		
Organ and findings	**Control**	**10% PP**	**PP–CPC (0.1% PP and 0.1% CPC)**	**Control**	**10% PP**	**PP–CPC (0.1% PP and 0.1% CPC)**
(Liver)						
Hepatocyte necrosis	–	–	–	–	–	1
Mononuclear cell infiltration	–	–	–	1	–	–
Hypermitotic activity	–	–	–	1	1	2
(Kidney)						
Basophilic tubules	1	1	1	–	1	1
Glassy degeneration	–	1	–	1	1	–
Cysts	–	1	–	–	–	–
Calcification	2	–	1	1	1	–
Collecting-tubule epithelial hypertrophy	–	–	6	–	–	2
Distal-tubular epithelial hypertrophy	–	–	–	–	–	2
(Ovary)						
Cysts				–	–	1
(Hematopoietic system)						
Extramedullary hyperplasia	–	–	–	–	1	–
(Respiratory system)						
Bronchiolar alveolar adenoma	–	–	–	1	–	–

Table 1. Incidence of histopathological findings in the GLP-compliant 14-day repeated-dose oral toxicity study in mice. Values indicate the number of animals showing each histopathological finding in the 14-day repeated-dose oral toxicity study in mice. Mice received water for injection, 10% phosphorylated pullulan (PP), or PP–CPC containing 0.1% PP and 0.1% cetylpyridinium chloride (CPC) once daily by oral gavage. The table summarizes incidence data; semiquantitative severity grades were not provided in the archived report for these findings. –, no animals with the finding. $n = 6$ per sex per group. Control, water for injection.

tissue accumulation vary substantially according to molecular structure, route of administration, and exposure conditions, with the kidneys identified as one of the organs in which these compounds may be distributed or retained[25,26]. However, these studies involved a different compound and exposure conditions that were not directly comparable with those used in the present study. The renal findings should therefore be interpreted cautiously, as their toxicological relevance and relationship to PP–CPC treatment remain uncertain. Although these findings were not accompanied by detectable increases in serum creatinine or urea nitrogen, conventional serum biochemical markers may not be sufficiently sensitive to detect mild, focal, or early renal structural changes[27,28].

In contrast to the systemic findings, the hamster buccal-pouch study provided local tolerability data directly relevant to oral mucosal exposure. Using predefined macroscopic and histopathological 0–4 grading criteria, no erythema, eschar formation, epithelial erosion, vascular congestion, or edema was observed in any group. Very slight/minimal leukocyte infiltration was observed only in the CPC-treated group, whereas no such finding was observed in the PP or PP–CPC groups. Importantly, no mild, moderate, or severe mucosal irritation was detected, as in human clinical study with 0.05% CPC[29]. These findings suggest that PP–CPC did not induce biologically meaningful buccal mucosal irritation under the tested conditions.

Inspection method	Findings	1% PP		0.05% CPC		PP–CPC (0.05% PP and 0.05% CPC)	
		Right cheek treated	Left cheek untreated	Right cheek treated	Left cheek untreated	Right cheek treated	Left cheek untreated
Macroscopic examination	Erythema	–	–	–	–	–	–
	Eschar formation	–	–	–	–	–	–
Histopathological examination	Epithelial changes	–	–	–	–	–	–
	Leukocyte infiltration	–	–	±	±	–	–
	Vascular congestion	–	–	±	–	–	–
	Edema	–	–	–	–	–	–

Table 2. Buccal mucosal irritation findings in the GLP-compliant study in hamsters. Male hamsters received once-daily topical application of 1% phosphorylated pullulan (PP), 0.05% cetylpyridinium chloride (CPC), or PP–CPC containing 0.05% PP and 0.05% CPC to the right buccal pouch for 4 consecutive days. The untreated left buccal pouch served as an internal control. Macroscopic and histopathological findings were evaluated approximately 24 h after the final application. Symbols indicate the maximum grade observed among five hamsters for each buccal pouch within each treatment group. Macroscopic findings were graded from 0 to 4 according to the predefined criteria for erythema and eschar formation. Histopathological findings were graded from 0 to 4 according to the predefined criteria for epithelial changes, leukocyte infiltration, vascular congestion, and edema. –, grade 0, absent; ±, grade 1, minimal; +, grade 2, mild; ++, grade 3, moderate; +++, grade 4, severe. $n = 5$ per group. The untreated left buccal pouch served as the internal control.

The microbiota-related analyses should be considered exploratory. In the rat oral microbiota analysis, total bacterial abundance and community profiles were assessed in-house using predefined real-time PCR and T-RFLP procedures. Although no marked change in relative total bacterial abundance was detected, T-RFLP profiling suggested treatment- and time-dependent differences in community patterns. Because T-RFLP does not provide species-level resolution[30,31] and the analysis was not conducted under blinded conditions, these findings should not be interpreted as definitive evidence of beneficial or controlled modulation of the microbiota. In the mouse study, fecal microflora analysis was performed only in male mice as an exploratory adjunct assessment; therefore, sex-specific effects and changes in female mice could not be evaluated. In addition, the mouse fecal analysis was culture-based and therefore limited to cultivable bacterial groups detectable under the applied conditions[32].

This study has several limitations. First, animal numbers were based on applicable testing guidelines and approved study protocols rather than formal effect-size-based power calculations, and the in-house rat study was exploratory. The in-house rat study was conducted in two sequential experimental phases with different animals and sample sizes for general toxicity and oral-microbiota assessments. Blood biochemical analyses combined samples obtained from animals in both phases, whereas external histopathological evaluation was limited to one animal of each sex per treatment group. Second, the mouse histopathological findings were recorded as detailed descriptive findings and incidence data, but semiquantitative severity grades were not provided in the archived report. Third, although the BioSafety Research Center studies were conducted under GLP-compliant conditions with individually identified animals and designated study personnel, blinding of histopathological evaluators was not explicitly documented in the archived reports. Fourth, microbiota analyses were exploratory; the rat oral microbiota analysis was performed in-house without blinding, and the mouse fecal microflora analysis was limited to male mice. Finally, the study did not include recovery groups, long-term exposure beyond the tested period, urinary biomarkers of renal injury, or species-level microbiome sequencing.

Taken together, the present findings support further development of PP–CPC as a candidate oral material, but they do not, by themselves, establish clinical safety or efficacy. The absence of overt systemic toxicity, the lack of biologically meaningful buccal mucosal irritation, and the limited cytotoxicity of PP itself provide a basis for carefully monitored early-phase clinical studies. Such studies should assess oral mucosal safety, adverse events, taste disturbance, tooth staining[10–12], renal function (including urinalysis, where appropriate), and changes in the oral microbiome, as well as exploratory clinical endpoints such as plaque accumulation, gingival inflammation[14,15,29], and CPC retention at the target site[18]. Future studies with larger sample sizes, longer recovery periods, and higher-resolution microbiome analyses will be necessary to more precisely define the safety margin and clinical utility of PP–CPC.

Materials and methods

Test compounds

Cationic cetylpyridinium chloride (CPC; Sigma, St. Louis, MO, USA) was used as the antibacterial agent. Phosphorylated pullulan (PP) was used as a carrier polymer to retain CPC on tooth surfaces and thereby prolong its antibacterial effect. PP was synthesized by KNC Laboratories Co., Ltd. (Kobe, Japan) by phosphorylating pullulan supplied as a Japanese Pharmacopoeia-grade material (Hayashibara Co., Ltd., Okayama, Japan), as described previously[18] with minor modifications. The PP used in this study was obtained from a single characterized lot (Lot No. 209-003-36-6; prepared in September 2009). According to the internal characterization record, the peak-top, weight-average, and number-average molecular weights were 23,000, 206,000, and 22,000, respectively. The sodium and phosphorus contents were 2.04 wt% and 3.39 wt%, respectively.

For the in vitro experiments, CPC and PP stock solutions were prepared in sterile Dulbecco's phosphate-buffered saline without calcium and magnesium [PBS(–); Gibco, Thermo Fisher Scientific, Waltham, MA, USA] to final concentrations of 0.1% or 0.2% (w/v), depending on the experiment, and diluted with sterile PBS to the required working concentrations. Immediately before use, PP and CPC solutions were combined to prepare PP–CPC solutions at the specified final concentrations. For the animal studies, test solutions were prepared in purified or ultrapure water as described in the respective experimental sections. No additional sterilization was performed after preparation.

In vitro cytotoxicity assays

Cells and culture

Three human cell types were used: the HeLa human cervical carcinoma cell line (American Type Culture Collection [ATCC]), the KB human oral carcinoma cell line (ATCC), and human gingival fibroblasts (HGFs; ScienCell, Carlsbad, CA, USA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 10 µg/mL gentamicin reagent solution at 37 °C in a humidified atmosphere containing 5% CO_2.

When cultures reached subconfluence, the cells were passaged to reduce cell density by approximately fourfold. For HGFs, cultures between passages four and nine were used in subsequent experiments. To evaluate the changes in cellular activity, cells were cultured for 24 h after inoculation in a medium containing 0.5% FBS. This low-serum condition was used to synchronize the cells at the quiescent stage before analysis.

Observation of cell morphology and counts

An optical microscope (ECLIPSE TS 100; Nikon, Shinagawa, Tokyo, Japan) and an Array Scan VTI HCS Reader (Thermo Fisher Scientific, Waltham, MA, USA) were used to observe the effects of the test solutions on HeLa cells. For phase-contrast optical observations, HeLa cells were seeded in a 12-well plate (Corning, Corning, NY, USA) at a density of 5.2×10^3 cells/cm^2. After cultivation using a previously described method, the cells were exposed to 0.01% and 0.1% test solutions (PP and PP–CPC solutions). Immediately before adding the test solutions, the culture medium was aspirated and rinsed twice with PBS. Next, the cultures were treated with each test solution for 3 min. Afterward, the cultures were rinsed twice with PBS, supplemented with culture medium, incubated for 24 h, and morphological changes were observed using an optical microscope (ECLIPSE TS 100).

For immunofluorescence (HeLa only) and cell counts (HeLa, KB, and HGFs), cells were seeded into a 48-well plate (Corning) at a density of 2.1×10^4 cells/cm^2. After cultivation using a previously described method, the cells were exposed to serial dilutions of the test solutions ranging from 0.0001% to 0.1% (PP and PP–CPC solutions). Similar concentrations of CPC and PBS were used as the controls. Treatment was performed using the procedure described above, and the cells were cultured for 24 h post-treatment. The culture medium was then removed, and the cells were rinsed with PBS, followed by fixation in PBS containing 4% paraformaldehyde (PFA) for 15 min. After fixation, the cells were rinsed three times with PBS and soaked for 5 min in PBS containing 0.1% Triton X-100 (Nacalai Tesque, Kyoto, Kyoto, Japan). The samples were rinsed three more times with PBS, then incubated with AlexaFluor® 594-phalloidin (Invitrogen) diluted 1:200 in PBS for 30 min at room temperature in the dark. To stain the nuclei, DAPI (ProLong® Gold and Slowfade® Gold Antifade Reagents with DAPI; Invitrogen) was added at room temperature for 10 min. Finally, the stained cells were observed and counted using an Array Scan VTI HCS Reader.

Cell viability

The viabilities of HeLa, KB, and HGFs following exposure to the test solutions were evaluated using the MTT assay[33,34], as previously described. After the cells were cultured under the conditions described above, MTT reagent (Sigma) was added to a final concentration of 0.5 mg/mL. After incubation for 4 h, the resulting bluish–brown formazan crystals formed by the metabolically active cells were dissolved in acidified propanol, and the dissolved solution was collected. The absorbance was measured at 595 nm using a Model 680 Microplate Reader (Bio-Rad Laboratories; Hercules, CA, USA).

In vivo repeated oral administration toxicity tests in rats

The following experiments evaluated systemic effects and potential changes in the oral microbiota during repeated oral administration of PP, CPC, or PP–CPC. This study was approved by the Animal Care and Use Committee of Okayama University (OKU-2010105). Humane endpoints were predefined for all in vivo experiments in accordance with institutional and national animal care guidelines. The animals were monitored at least once daily for clinical signs of pain or distress. However, none of the animals met the criteria for humane endpoints during the course of the study. A total of 40 Sprague–Dawley rats (20 males and 20 females), aged 4 weeks, were obtained from Charles River Japan (Yokohama, Kanagawa, Japan). The animals were housed at the designated specific-pathogen-free Okayama University Animal Facility, which maintained a room temperature of 22–24 °C and artificial lighting from 06:00 to 18:00 h. Rats were housed individually throughout the study, allowing daily water consumption and intake of the assigned solution to be monitored for each animal. During the study, the rats had free access to radiation-sterilized solid feed (CE-2; CLEA Japan Inc., Meguro, Tokyo, Japan). The rats were allocated to four treatment groups, with five males and five females in each group. The study was conducted in two sequential experimental phases. In the first phase, three males and three females per treatment group were used for general toxicity assessment, including clinical observations, body-weight and intake measurements, blood sampling, necropsy, and tissue collection. In the second phase, initiated approximately 2 months later, two additional males and two additional females per treatment group were used for longitudinal oral-microbiota assessment, with oral samples collected at baseline and at weeks 2 and 4. No analgesic or other pain-relief interventions were implemented during the study.

The concentration of the test solutions was set to 0.01%, which corresponds to the levels anticipated for clinical use. CPC and PP stock solutions were diluted to 0.1% with ultrapure water. A PP–CPC solution containing 0.01% CPC and 0.01% PP was prepared by mixing equal volumes of 0.02% CPC and 0.02% PP solutions. Rats in each experimental phase were assigned to four groups: ultrapure water, 0.01% PP, 0.01% CPC, or PP–CPC (0.01% PP and 0.01% CPC). The assigned test or control solution was provided as the sole drinking fluid throughout the 28-day administration period.

During the first experimental phase, all 24 rats were monitored daily for mortality and visible abnormalities. Body weight was recorded weekly, and food and water intake were measured daily in three animals of each sex per treatment group. Each day, the water container and feed dispenser were replaced, and the remaining water and food were weighed. Intake was calculated from the difference between the amounts supplied and the amounts remaining.

Necropsy and blood sampling

At the end of the observation period, the 24 rats used for general toxicity assessment were sedated with diethyl ether diluted 1:2 with physiological saline and subsequently anesthetized by intraperitoneal administration of Nembutal (0.1 mL/100 g body weight; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), in accordance with the approved institutional protocol in effect at the time of the study. Under anesthesia, an incision was made on the ventral surface to expose the heart, and blood collection was attempted from the heart using a 22-gauge needle for exsanguination. Three animals from each treatment group underwent transcardial perfusion fixation with 4% PFA. From these perfusion-fixed animals, one male and one female per treatment group were prespecified from among the animals used in the first experimental phase for external histopathological evaluation, resulting in a total of eight rats. The liver, kidneys, spleen, and gingival tissues were excised and immediately fixed in 4% PFA[35]. At the end of the second experimental phase, after completion of the week-4 oral sampling, blood was also collected from the animals used for oral-microbiota assessment.

Blood biochemical examination

Blood biochemical analyses were performed using samples from four animals of each sex per treatment group by FALCO Biosystems Ltd. (Kyoto, Kyoto, Japan). The analyzed samples comprised two animals of each sex per group from the first experimental phase and two animals of each sex per group from the subsequent oral-microbiota phase. The following parameters were measured: total bilirubin, total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglycerides, sodium, chloride, potassium, calcium, inorganic phosphorus, urea nitrogen, creatinine, white blood cell count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, reticulocytes, neutrophils, eosinophils, basophils, lymphocytes, and monocytes[35].

Histopathological examination

Histopathological examination of tissue specimens from the eight prespecified rats was outsourced to Biopathology Institute Co., Ltd. (Kunisaki, Oita, Japan). Tissue specimens were submitted using specimen identification numbers only, without treatment-group information; therefore, the external evaluator was blinded to treatment allocation. The liver, kidneys, spleen, and gingival tissues from each animal were examined microscopically. Microscopic findings were described using diagnostic terms in the contract report. A final summary table categorized the findings using symbolic descriptors: −, no abnormality; ±, very slight change; +, mild change; and ++, moderate change.

Analysis of the oral microbiota in rats

Bacteria were periodically collected from the oral cavity to perform an exploratory in-house analysis of potential changes in oral microbial abundance and community structure during repeated administration. Genomic DNA was extracted from the collected samples, and total bacterial abundance was quantified using real-time polymerase chain reaction (PCR). Community profiles were assessed using terminal restriction fragment length polymorphism (T-RFLP) analysis followed by hierarchical clustering. These analyses were conducted according to predefined laboratory procedures. Blinding to treatment allocation was not implemented during these in-house microbiota analyses.

The second experimental phase was initiated approximately 2 months after completion of the general toxicity assessment. Oral bacterial samples were collected from two males and two females per treatment group at baseline and at weeks 2 and 4 ($n = 4$ per group). The same animals were sampled longitudinally throughout the 4-week period. During sampling, each rat was gently restrained, and an autoclaved Benda Micro brush (J. MORITA CORP., Suita, Osaka, Japan) was inserted into the oral cavity. The buccal surface of the left molar was wiped five times with a brush, and the collected material was transferred into a 1.5 mL microtube containing 1 mL of PBS. The brush was agitated in PBS for 1 min to create a turbid suspension, which was then transferred into separate microtubes. Samples were centrifuged at 10,000×*g* for 15 min at 4 °C, after which the supernatant was discarded. The resulting pellet was resuspended in 200 µL of InstaGene™ Matrix (BIO-RAD Laboratories) and stored at − 30 °C until further analysis.

Oral bacterial DNA quantification

For total DNA extraction, the frozen samples were heated at 56 °C for 30 min and then heated at 100 °C for 8 min. The supernatant (50 µL) was recovered by centrifugation and used as the DNA template. Relative bacterial abundance was quantified using real-time PCR targeting the bacterial 16 S rRNA gene, with relative quantification based on the ΔCt method[36,37]. One-twentieth of the extracted DNA sample was combined with

12.5 µL of the SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA), containing AmpliTaq Gold® DNA Polymerase (Thermo Fisher Scientific)[38], 2 µL each of forward (5′-GTGSTGCAYGGYTGTCGTCA-3′) and reverse (5′-ACGTCRTCCMCACCTTCCTC-3′) primers (10 µM each; Sigma), and nuclease-free water (Qiagen GmbH, Hilden, Germany) to a final reaction volume of 25 µL. PCR amplification was initiated with denaturation at 95 °C for 10 min, followed by 35 cycles of 15 s at 95 °C and 1 min at 60 °C. Fluorescence signals were measured using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems).

Analysis of oral microbiota using T-RFLP

Bacterial 16 S rRNA gene fragments were amplified using a PCR protocol with universal primers (forward 27 F: 5′-AGAGTTTGATCCTGGCTGCTCAG-3′; reverse 1492R: 5′-GGTTACCTTGTTACGACTT-3′; Sigma). The 27 F forward primer was labeled at the 5′ end with 6-carboxyfluorescein (6-FAM) in accordance with the T-RFLP protocol[30,39]. PCR reactions were conducted using 2–5 µL of the extracted DNA as template, supplemented with 0.25 µL of TaKaRa Ex Taq DNA polymerase (5 U/µL; Takara Bio Inc., Kusatsu, Shiga, Japan), 5 µL of 10 × Ex Taq buffer, 4 µL of 2.5 mM dNTP mixture, 10 pmol of the fluorescently labeled forward primer, 10 pmol of the reverse primer, and RNase-free water to a final volume of 50 µL. Amplification was performed using an ASTEC Program Temp Control System PC-700 (ASTEC Co., Ltd., Fukuoka, Japan) under the following cycling conditions: 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 90 s, followed by a final extension step at 72 °C for 10 min. The PCR products were verified by agarose gel electrophoresis. Aliquots (2 µL) of the amplified products were separated on 1.5% agarose gels prepared in the TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide (final concentration, 50 µg/mL), and visualized under ultraviolet illumination (260 nm).

The PCR products were purified using a MiniElute PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Briefly, five volumes of Buffer PB were added to the PCR mixture, followed by centrifugation for 1 min. After the addition of 750 µL of Buffer PE, samples were centrifuged again, and the purified DNA was eluted with 10 µL of Buffer EB. The eluted PCR products were recovered in a final volume of 9 µL. Purified PCR products were subsequently digested with the restriction enzyme MspI (Takara Bio, Inc.) to generate terminal restriction fragments. The digestion mixture consisted of 2 µL of purified PCR product, 2 µL of MspI (20 U/µL), 1 µL of bovine serum albumin (1%), the appropriate reaction buffer, and RNase-free water to a final volume of 10 µL. Restriction digestion was performed at 37 °C for 3 h.

Restricted PCR products (1 µL) were denatured to single-stranded DNA by incubation at 95 °C for 2 min, followed by immediate cooling on ice. The denaturation mixture consisted of 1 µL of restricted PCR product, 12 µL of deionized formamide, and 1 µL of DNA fragment length standard. The fragment length standard was prepared by mixing GS 500 ROX and GS 1000 ROX size standards (Applied Biosystems) in a 1:1 ratio. Terminal restriction fragment lengths were determined using the GeneScan mode on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) under the following conditions: 15 kV, 8 mA, 60 °C, and 60 min per sample. Fragment analysis was performed using the GeneScan software version 3.1 (Applied Biosystems). Fluorescent peaks with intensities below 50 relative fluorescence units were excluded as background noise, in accordance with the established T-RFLP analytical criteria using standard procedures for T-RFLP analysis.

Hierarchical clustering analysis

Hierarchical clustering analysis was performed using the MicrobiotaProfiler software (Infocom, Minato, Tokyo, Japan) based on T-RFLP data from all samples, and dendrograms were generated to visualize similarities in oral microbial community structures[32].

In vivo toxicity studies in mice and hamsters under GLP conditions

The mouse single-dose and repeated-dose oral toxicity studies and the hamster buccal-pouch mucosal irritation study were conducted by the BioSafety Research Center (BSRC; Iwata, Shizuoka, Japan) under GLP-compliant conditions according to the approved study protocols. Animals were individually housed and identified by animal numbers. Animal care, dosing, necropsy, and histopathological examinations were performed by designated personnel at the facility. Pathological findings were recorded in detail for each animal, and pathological data were managed using the PATHOTOX integrated pathology and toxicology data system where applicable.

In parallel, potential effects on fecal microflora were assessed by the Flora Service Japan Corp. (Kokubunji, Tokyo, Japan). These culture-based fecal microflora analyses were performed as exploratory adjunct assessments and were not included as GLP study endpoints.

The animal study protocols were reviewed and approved by the Animal Study Committee of BSRC: protocol no. 10–0138 A for the single-dose study (study no. C757), protocol no. 10–0216 A for the repeated-dose study (study no. D053), and protocol no. 11–0079 A for the buccal-pouch mucosal irritation study (study no. D604). These protocols were prepared before initiation of the respective GLP studies but were not registered in a publicly accessible protocol repository. None of the animals were genetically modified or had undergone previous experimental procedures before enrollment in the respective studies. Throughout the externally conducted GLP studies, general health and clinical signs were monitored in accordance with the respective study protocols. Anesthesia was used for terminal procedures: mice were anesthetized with diethyl ether before euthanasia by exsanguination, and hamsters were euthanized by pentobarbital overdose.

Repeated-dose oral toxicity study in mice

A total of 36 Slc: ICR mice (18 males and 18 females; $n = 6$ per sex per group) were obtained from Japan SLC, Inc. (Shizuoka, Japan) at 5 weeks of age for the repeated-dose oral toxicity study. After a 1-week quarantine and acclimatization period, the animals were housed in a barrier-system room maintained at 23–26 °C under

controlled lighting conditions (07:00–19:00). The mice were provided ad libitum access to radiation-sterilized solid feed (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water supplied via an automatic nozzle system throughout the study. The administration and observation periods were set at 14 days, during which the general condition of all animals was monitored once daily, and body weights were recorded at weekly intervals.

Test solution concentrations were determined based on the established repeated-dose toxicity testing guidelines. The PP solution was administered at 10% (w/v), corresponding to 1,000 μg/g body weight, which is generally regarded as the upper limit for repeated oral administration toxicity studies in mice. The PP–CPC solution was prepared at a concentration of 0.1% (w/v) for each component, which is consistent with the concentrations evaluated in previous in vitro studies. Water for injection was administered to mice in the vehicle-control group. The mice received the assigned test solutions once daily by oral gavage using a disposable syringe fitted with a gastric feeding tube. Dosing volumes were calculated at 10 μL/g body weight based on the most recent body weight measurements.

Culture-based analysis of fecal microflora

Fecal microflora analysis was performed as an exploratory adjunct assessment in male mice only ($n = 6$ per group). Male mice were selected to reduce biological variability related to sex and the estrous cycle and to limit the number of animals and analyses, because this analysis was not a primary endpoint of the GLP toxicity study. Fecal microflora was examined using culture-based methods with non-selective and selective agar media. Non-selective media were used to assess cultivable fecal bacteria, and selective media were used to evaluate major bacterial groups, including *Lactobacillus*, *Bacteroidaceae*, *Enterobacteriaceae*, *Bifidobacterium*, and lecithinase-positive clostridia. Bacterial counts were recorded when colonies were detected under the applied culture conditions.

Fecal samples (> 0.1 g) were collected from male mice at the start of the experiment and at necropsy following the completion of the dosing period. Samples were placed in ceramic tubes, and an anaerobic dilution solution (provided by Flora Service Japan Corp.) was added to obtain a tenfold dilution of the fecal material. The samples were immediately frozen and transported under freezing conditions to Flora Service Japan Corp., arriving in the morning following each collection.

Fecal microflora was analyzed according to the Mitsuoka method[32]. Briefly, samples were further diluted 100-fold with anaerobic diluent, and 0.05 mL of each dilution was inoculated onto one-third to one-quarter of the surface of the appropriate agar media. The bacterial populations were identified based on colony morphology, Gram staining, and cellular morphology. Colony counts were adjusted according to the dilution factor and expressed as logarithmic values per gram of fecal material.

Glucose–blood–liver (BL) agar and Eggerth–Gagnon (EG) agar were used as non-selective media. Selective media included neomycin–Nagler (NN) agar for lecithinase-positive clostridia, *Bifidobacteria*-selective (BS) agar for *Bifidobacterium*, lactobacilli selective (LBS) agar for *Lactobacillus*, neomycin–brilliant green–taurocholate–blood (NBGT) agar for Bacteroidaceae, and deoxycholate-hydrogen sulfide–lactose (DHL) agar for Enterobacteriaceae.

Hematological and biochemical examination

At the end of the observation period, the mice were anesthetized with diethyl ether in accordance with the approved study protocol. Under anesthesia, blood samples were collected from the abdominal aorta for hematological and biochemical analyses, and the animals were euthanized by exsanguination. Hematological and biochemical analyses were performed at the outsourcing facility according to the approved study protocol.

Hematological parameters were measured using a complete hematology system (ADVIA 120; Bayer, Tokyo, Japan). Hematological parameters included white blood cell count, red blood cell count, hemoglobin, hematocrit, MCV, MCH, MCHC, platelet count, reticulocytes, neutrophils, eosinophils, basophils, lymphocytes, and monocytes.

The biochemical parameters analyzed included total bilirubin, total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglycerides, sodium, chloride, potassium, calcium, inorganic phosphorus, urea nitrogen, and creatinine.

Pathological examination

Following exsanguination, gross necropsy was performed, and the major organs, including the heart, liver, kidneys, adrenal glands, testes, epididymides, ovaries, uterus, lungs, thymus, and brain, were weighed.

For the histopathological examination, the heart, kidneys, jejunum, and distal colon were excised and fixed in 10% neutral-buffered formalin. The fixed tissues were processed using standard paraffin embedding procedures, sectioned, stained with hematoxylin and eosin, and examined microscopically.

Histopathological findings were recorded descriptively for each animal. The findings summarized in Table 1 are presented as the number of animals showing each microscopic finding in each group. Because the archived report did not provide semiquantitative severity grades for the findings listed in Table 1, the table presents incidence data rather than graded severity scores.

Single-dose oral toxicity study in mice

For the single-dose oral toxicity study, a total of 30 Slc: ICR mice (15 males and 15 females; $n = 5$ per sex per group) were obtained from Japan SLC, Inc. (Shizuoka, Japan) at 5 weeks of age. After a 1-week quarantine and acclimatization period, the animals were housed in a barrier-system room maintained at 23–26 °C under controlled lighting conditions (07:00–19:00). Throughout the study, the mice were provided ad libitum access to radiation-sterilized solid feed (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water supplied via an automatic nozzle system.

The test solution concentrations were determined based on established toxicity testing guidelines. The PP solution was administered at 20% (w/v), corresponding to 2,000 μg/g body weight, as the high-dose level specified in the approved single-dose study protocol. The PP–CPC solution contained 0.1% PP and 0.1% CPC, representing a tenfold increase relative to the concentration used in the repeated-dose study. Water for injection was administered to the vehicle-control group of mice. Following a 4-h fasting period, the mice received a single oral dose of the assigned test solution via a disposable syringe fitted with a gastric gavage tube. Dosing volumes were calculated at 10 μL/g body weight based on the most recent body weight measurements.

Animals were observed individually for clinical signs within 30 min after dosing and subsequently at hourly intervals for the first 4 h. Observations were conducted once daily the following day. Body weight was recorded weekly during the 14-day observation period. At the end of the 14-day observation period, the mice were anesthetized with diethyl ether and euthanized by exsanguination in accordance with the approved study protocol. Gross necropsy was then performed. The external surfaces, natural orifices, and organs within the abdominal, thoracic, pelvic, and cranial cavities were examined macroscopically. The major organs, including the heart and lungs, were removed during necropsy. Any gross abnormalities were recorded, and the affected organs or tissues were fixed in 10% neutral-buffered formalin for further evaluation.

Buccal-pouch mucosal irritation study in hamsters

A total of 15 male Syrian hamsters (Slc: Syrian) were obtained from Japan SLC, Inc. (Shizuoka, Japan) at 7 weeks of age for the buccal-pouch mucosal irritation study. After a 1-week quarantine and acclimatization period, the animals were housed in a barrier-system room maintained at 23–26 °C under controlled lighting conditions (07:00–19:00). The hamsters were provided ad libitum access to radiation-sterilized solid feed (CRF-1; Oriental Yeast Co., Ltd., Itabashi, Tokyo, Japan) and tap water supplied via water bottles throughout the study.

Test solution concentration and administration method

The test solution concentrations were determined based on previous in vitro findings and their clinical relevance. The PP solution was prepared at 1% (w/v), which corresponded to a 100-fold increase relative to the concentration used in previous in vitro studies (0.01%). The PP–CPC solution contained 0.05% PP and 0.05% CPC, whereas the CPC solution contained 0.05% CPC, which represents the upper concentration limit of commercially available quasi-drug CPC products in Japan.

Administration procedure

The test solutions were applied topically to the buccal-pouch mucosa ($n = 5$ per group). The animals were gently restrained, and the oral cavity was opened using silicone rubber-coated forceps to carefully expose the buccal pouch without causing tissue damage. The mucosal surface was rinsed with physiological saline, and the absence of visible abnormalities, such as inflammation, was confirmed before application.

A volume of 0.2 mL of the test solution was applied to the right buccal pouch using a 1 mL disposable syringe. This procedure was performed once daily for 4 consecutive days. The left buccal pouch was not treated and served as an internal control. Body weight was recorded at the time of initial administration and before necropsy. The general behavior was observed immediately before each administration and approximately 24 h after the final dose.

Macroscopic examination of buccal mucosa

The buccal-pouch mucosa was examined macroscopically immediately before each administration and approximately 24 h after the final dose. The presence or absence of lesions, including erythema, edema, or ulceration, was assessed by unaided visual inspection. Erythema and eschar formation were evaluated according to predefined 0–4 grading criteria in the contract study protocol: 0, no erythema; 1, very slight erythema; 2, well-defined erythema; 3, moderate erythema; and 4, severe erythema or eschar formation that interfered with erythema grading. Other undesirable changes, if present, were recorded descriptively.

Histopathological examination

After completion of the final macroscopic examination, the hamsters were euthanized by an overdose of pentobarbital in accordance with the approved study protocol. Buccal-pouch tissues from the treated right side and the untreated left side (as an internal control) were excised, fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and stained with H&E for microscopic examination. Histopathological findings were evaluated using predefined 0–4 grading criteria in the contract study protocol for epithelial changes, leukocyte infiltration, vascular congestion, and edema. Epithelial changes were graded from 0 (normal, intact epithelium) to 4 (diffuse erosion). Leukocyte infiltration was graded by the number of leukocytes per high-power field at ×400 magnification: 0, absent; 1, minimal (< 25 cells); 2, mild (26–50 cells); 3, moderate (51–100 cells); and 4, marked (> 100 cells). Vascular congestion and edema were each graded from 0 (absent) to 4 (marked).

Animal allocation

Formal randomization was not performed. For all animal studies, animals were removed from their home cages without reference to baseline characteristics and were systematically allocated to treatment groups according to that sequence to reduce selection bias. No stratification according to baseline characteristics was performed. Potential confounders related to treatment order and measurement order were not specifically controlled.

Blinding

In the in-house rat study, personnel responsible for animal allocation, preparation and administration of the test solutions, and routine observations were aware of the treatment assignments. The external

histopathological evaluator was blinded to treatment allocation because tissue specimens were submitted using identification numbers without treatment-group information. Blinding was not implemented during the in-house oral-microbiota analyses. For the externally conducted GLP studies in mice and hamsters, the archived reports indicated that designated personnel performed animal care, dosing, necropsy, and histopathological examinations; however, they did not explicitly document whether personnel at these experimental stages or during data analysis were blinded to treatment allocation.

Inclusion and exclusion criteria

No prespecified criteria were established for excluding animals or experimental units from any of the animal studies. For the T-RFLP analysis, fluorescent peaks with intensities below 50 relative fluorescence units were excluded as background noise according to the predefined analytical criterion. No animals were excluded from any analysis. Three oral-microbiota samples—CPC-2 at week 2, Control-2 at week 4, and Control-4 at week 4—were unavailable and were therefore excluded from the T-RFLP analysis. All other collected data were included in the corresponding analyses.

Sample size

No formal, study-specific statistical power calculation was performed for any of the animal studies.

For the in-house 28-day rat drinking-water study, five males and five females were assigned to each treatment group. The study was conducted in two sequential experimental phases. In the first phase, three animals of each sex per group were used for general toxicity assessment, including clinical observations, body-weight and intake measurements, blood collection, necropsy, and tissue collection. Approximately 2 months later, two additional animals of each sex per group were used for longitudinal oral-microbiota assessment. Blood biochemical data were available from four animals of each sex per group, comprising samples obtained from both experimental phases. External histopathological evaluation was performed in one male and one female from each treatment group selected from the first phase. This study was exploratory and supportive in nature and was intended to identify overt treatment-related changes and exploratory microbiota alterations rather than to exclude small, infrequent, or sex-specific effects. No formal a priori sample size calculation was performed because the animal numbers were determined according to the respective study objectives, relevant guidelines, or predefined protocols. Accordingly, no single primary outcome measure was prespecified or used to determine the sample size because the study comprised multiple exploratory and guideline-based safety assessments.

For the externally conducted GLP-compliant studies, the numbers of animals were predefined in the approved study protocols according to the applicable testing guidelines. The repeated-dose mouse study used six animals of each sex per group and was designed with reference to OECD Test Guideline 407. The single-dose mouse study used the number of animals specified in the prespecified GLP study protocol for acute oral toxicity assessment. For the hamster buccal-pouch mucosal irritation study, the number of animals was determined according to the prespecified GLP study protocol with reference to ISO 7405 and ISO 10993-10 for dental-material mucosal irritation testing.

A total of 121 animals were used across the four in vivo experiments: 40 rats in the 28-day study conducted in two sequential phases (24 in the first phase and 16 in the second phase), 36 mice in the repeated-dose study, 30 mice in the single-dose study, and 15 hamsters in the buccal-pouch mucosal irritation study.

Statistics

Data are presented as mean ± standard deviation, unless otherwise stated. Comparisons between two groups were performed using Student's *t*-test or Welch's *t*-test, as appropriate. Homogeneity of variance was assessed using the F-test, and Welch's *t*-test was used when variances were unequal. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by Scheffé's post-hoc test, as appropriate. Formal tests of normality were not performed because several analyses involved small sample sizes and exploratory safety assessments. No separate formal test of homogeneity of variance was performed for multiple-group comparisons. Statistical significance was set at a two-sided p-value < 0.05. Statistical analyses were performed using Excel for Mac (Microsoft, Redmond, WA, USA), IBM SPSS Statistics (IBM Corp., Armonk, NY, USA), and JMP version 9.0 (SAS Institute Inc., Cary, NC, USA). Because of the small sample sizes in some animal experiments, assessment of data distribution was limited. Therefore, nonsignificant results were interpreted with caution and not regarded as definitive evidence of the absence of biological effects.

Data availability

The datasets generated and analyzed in the current study are available from the corresponding author upon reasonable request.

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References

1. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322 (1999).
2. Kassebaum, N. J. et al. Global, regional, and national prevalence, incidence, and disability-adjusted life years for oral conditions for 195 countries, 1990–2015: a systematic analysis for the global burden of diseases, injuries, and risk factors. *J. Dent. Res.* **96**, 380–387 (2017).
3. GBD. Trends in the global, regional, and national burden of oral conditions from 1990 to 2021: a systematic analysis for the Global Burden of Disease Study 2021. *Lancet* **405**, 897–910 (2025).

4. Okamoto, E. Japan's dental care facing population aging: how universal coverage responds to the changing needs of the elderly. *Int. J. Environ. Res. Public. Health*. **18**, 9359 (2021).
5. Kocher, T. et al. Trends in periodontal status: results from the German Oral Health studies from 2005 to 2023. *Quintessence Int.* **56**, S48–S58 (2025).
6. Kuang, X., Chen, V. & Xu, X. Novel approaches to the control of oral microbial biofilms. *Biomed. Res. Int.* **2018**, 6498932, 2018. (2018).
7. Azzolino, D. et al. The emerging role of oral microbiota: a key driver of oral and systemic health. *Am. J. Dent.* **38**, 111–116 (2025).
8. Fardal, O. & Turnbull, R. S. A review of the literature on use of chlorhexidine in dentistry. *J. Am. Dent. Assoc.* **112**, 863–869 (1986).
9. Aldeen Salaymeh, E., Steinberg, D. & Abu Ammar, A. Chlorhexidine-loaded microneedles for treatment of oral diseases. *Int. J. Pharm.* **670**, 125143 (2025).
10. Zanatta, F. B., Antoniazzi, R. P. & Rösing, C. K. Staining and calculus formation after 0.12% chlorhexidine rinses in plaque-free and plaque covered surfaces: a randomized trial. *J. Appl. Oral Sci.* **18**, 515–521 (2010).
11. Kamble, A., Jabin, Z., Agarwal, N. & Anand, A. Effectiveness of oral probiotics in reducing *S. mutans* count in caries-active children: a comparison with chlorhexidine and herbal mouthrinse (Hiora®). *Int. J. Clin. Pediatr. Dent.* **15** (Supplement 2), S207–S211 (2022).
12. Okano, M. et al. Anaphylactic symptoms due to chlorhexidine gluconate. *Arch. Dermatol.* **125**, 50–52 (1989).
13. Roberts, W. R. & Addy, M. Comparison of the *in vivo* and *in vitro* antibacterial properties of antiseptic mouthrinses containing chlorhexidine, alexidine, cetyl pyridinium chloride and hexetidine. Relevance to mode of action. *J. Clin. Periodontol.* **8**, 295–310 (1981).
14. Haps, S., Slot, D. E., Berchier, C. E. & Van der Weijden, G. A. The effect of cetylpyridinium chloride-containing mouth rinses as adjuncts to toothbrushing on plaque and parameters of gingival inflammation: a systematic review. *Int. J. Dent. Hyg.* **6**, 290–303 (2008).
15. Aguilera, F. R. et al. Substantivity of mouth-rinse formulations containing cetylpyridinium chloride and O-cymen-5-ol: a randomized-crossover trial. *BMC Oral Health*. **22**, 646 (2022).
16. Nakanishi, K. et al. From tooth adhesion to bioadhesion: development of bioabsorbable putty-like artificial bone with adhesive to bone based on the new material phosphorylated pullulan. *Mater. (Basel)*. **17**, 3671 (2024).
17. Nakanishi, K. et al. New future dental material: antimicrobial material cetylpyridinium chloride-montmorillonite and implantable material phosphorylated pullulan. *Dent. Mater. J.* **44**, 1–8 (2025).
18. Namba-Koide, N. et al. Phosphorylated pullulan as a local drug delivery matrix for cationic antibacterial chemicals to prevent oral biofilm. *BMC Oral Health*. **25**, 1333 (2025).
19. Sreenivasan, P. & Gaffar, A. Antiplaque biocides and bacterial resistance: a review. *J. Clin. Periodontol.* **29**, 965–974 (2002).
20. Obeng, B. et al. Pharmaceutical agent cetylpyridinium chloride inhibits immune mast cell function by interfering with calcium mobilization. *Food Chem. Toxicol.* **179**, 113980 (2023).
21. Ma, L. et al. Adolescent cetylpyridinium chloride exposure impairs homologous recombination repair and induces granulosa cell apoptosis and follicular atresia via FOXM1/crebbp complex suppression. *Ecotoxicol. Environ. Saf.* **296**, 118193 (2025).
22. Wu, C. et al. Cetylpyridinium chloride disrupts maternal-to-zygotic transition during early embryonic development by impairing mitochondrial function and histone modification. *Ecotoxicol. Environ. Saf.* **302**, 118608 (2025).
23. Ministry of Health. Labour and Welfare of Japan. Approval Standards for Manufacturing and Marketing of Medicated Dentifrices (Notification No. 0628-3, 28 June 2021) (in Japanese). https://www.mhlw.go.jp/web/t_doc?dataId=00tc6011&dataType=1&pageNo=1 (Accessed 14 July 2026).
24. Müller, H. D., Eick, S., Moritz, A., Lussi, A. & Gruber, R. Cytotoxicity and antimicrobial activity of oral rinses in vitro. *Biomed. Res. Int.* **2017**, 4019723 (2017).
25. Xue, Y. et al. Distribution and disposition of benzalkonium chloride following various routes of administration in rats. *Toxicol. Lett.* **148**, 113–123 (2004).
26. Kera, H. et al. Kinetics and distribution of benzalkonium compounds with different alkyl chain length following intravenous administration in rats. *Leg. Med. (Tokyo)*. **48**, 101821 (2021).
27. Lock, E. A. Sensitive and early markers of renal injury: where are we and what is the way forward? *Toxicol. Sci.* **116**, 1–4 (2010).
28. Obert, L. A., Elmore, S. A., Ennulat, D. & Frazier, K. S. A review of specific biomarkers of chronic renal injury and their potential application in nonclinical safety assessment studies. *Toxicol. Pathol.* **49**, 996–1023 (2021).
29. Quirynen, M. et al. A 0.05% cetyl pyridinium chloride/0.05% chlorhexidine mouth rinse during maintenance phase after initial periodontal therapy. *J. Clin. Periodontol.* **32**, 390–400 (2005).
30. Sakamoto, M., Takeuchi, Y., Umeda, M., Ishikawa, I. & Benno, Y. Application of terminal RFLP analysis to characterize oral bacterial flora in saliva of healthy subjects and patients with periodontitis. *J. Med. Microbiol.* **52**, 79–89 (2003).
31. Nakano, Y. et al. Development and application of a T-RFLP data analysis method using correlation coefficient matrices. *J. Microbiol. Methods*. **75**, 501–505 (2008).
32. Mitsuoka, T. Establishment of intestinal bacteriology. *Biosci. Microbiota Food Health*. **33**, 99–116 (2014).
33. Gerlier, D. & Thomasset, N. Use of MTT colorimetric assay to measure cell activation. *J. Immunol. Methods*. **94**, 57–63 (1986).
34. Imazato, S. et al. Proliferation and differentiation potential of pluripotent mesenchymal precursor C2C12 cells on resin-based restorative materials. *Dent. Mater. J.* **29**, 341–346 (2010).
35. Omosu, M. et al. Oral repeated-dose toxicity studies and toxicokinetic studies of telithromycin in rats. *Jpn J. Chemother.* **51**, 103–114 (2003).
36. Maeda, H. et al. Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tetQ* gene and total bacteria. *FEMS Immunol. Med. Microbiol.* **39**, 81–86 (2003).
37. Okuda, H., Ohya, K., Shiota, Y., Kato, H. & Fukushi, H. Detection of *Chlamydophila psittaci* by using SYBR Green real-time PCR. *J. Vet. Med. Sci.* **73**, 249–254 (2011).
38. Morrison, T. B., Weis, J. J. & Wittwer, C. T. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques* **24**, 954–962 (1998).
39. Miyagawa, J. et al. Rapid and simple detection of eight major periodontal pathogens by the loop-mediated isothermal amplification method. *FEMS Immunol. Med. Microbiol.* **53**, 314–321 (2008).

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Declarations

Ethics approval and consent to participate
All animal experiments were conducted in compliance with the National Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Research Committee of Okayama University (approval no. OKU-2010105).

Consent for publication
Not applicable.

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The authors declare no competing interests.

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