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ORIGINAL ARTICLE

Bestatin as a treatment modality in experimental periodontitis

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Abstract

Background: Chronic periodontitis (CP), the most prevalent dysbiotic bacteria-driven chronic inflammatory disease, is an underestimated global health problem in itself, and due to a causative relationship with other disorders such as cardiovascular diseases or Alzheimer disease. The CP pathogenesis is primarily driven by *Porphyromonas gingivalis* in humans, and *Porphyromonas gulae* in dogs. These microorganisms initiate a pathogenic shift in the composition of the tooth-surface microflora. Our objective was to evaluate antimicrobial effects of bestatin, a potential CP drug candidate.

Methods: We evaluated bestatin bacteriostatic efficiency against periodontopathogens in planktonic cultures via microplate assay, and mono- and multispecies oral biofilm models. Neutrophil bactericidal activities, such as phagocytosis, were investigated in vitro using granulocytes isolated from the peripheral blood. The therapeutic efficacy and the immunomodulatory function of bestatin was assessed in a murine model of CP.

Results: Bestatin exhibited bacteriostatic activity against both *P. gingivalis* and *P. gulae*, and controlled the formation and species composition of the biofilm. We demonstrated that bestatin promotes the phagocytosis of periodontopathogens by neutrophils. Finally, we found that providing bestatin in the animal feed prevented alveolar bone resorption.

Conclusions: We show that in a murine model of CP bestatin not only shifted the biofilm species composition from pathogenic to a commensal one, but also promoted bacteria clearance by immune cells and alleviated inflammation. Taken together, these results suggest that bestatin is a promising drug choice for the treatment and/or prevention of periodontitis and clinical trials are required to fully evaluate its potency.

KEYWORDS
bestatin, biofilm, periodontitis, *Porphyromonas gingivalis*, *Porphyromonas gulae*

1 | INTRODUCTION

Chronic periodontitis (CP) is a bacteria-driven chronic inflammatory disease of the tissues surrounding teeth. The keystone pathogens, *Porphyromonas gingivalis* in humans and *Porphyromonas gulae* in canines, initiate a shift in the composition of the subgingival tooth-surface microflora from commensal to pathogenic.[1] *P. gingivalis* together with *Treponema denticola* and *Tannerella forsythia* constitute a group of pathogens termed the "red complex".[2] Proliferation of "red complex" species initiates chronic inflammation of the periodontium, which can persist for years and cause erosion of tooth-supporting structures. Globally, about 9% of people lose their teeth due to severe, untreated CP.[3] Signs of inflammation are visible not only locally in the periodontal pockets, but also systemically as elevated levels of inflammatory molecules in the blood of patients with periodontitis.[4] This systemic inflammation was demonstrated to promote development of atherosclerotic plaques, and aggravation of inflammatory diseases, including rheumatoid arthritis and Alzheimer disease (AD).[5] A study performed in 2017 found that 100% of patients with cardiovascular disease had *P. gingivalis* arterial colonization.[6] *P. gingivalis* and the immunoinflammatory host response in CP have also been identified as significant risk factors for developing amyloid β plaques, dementia, and AD,[7,8] contributing to the cognitive decline. *P. gingivalis*' virulence factors were detected in brain biopsies of patients suffering from AD, and their levels directly correlated with the levels of tau tangles.[9] Some studies have demonstrated a direct association between CP and certain forms of cancer.[10,11] *P. gingivalis* is detected in the dental plaques of $\approx$ 60%–90% of patients with CP, while *P. gulae* is detected at even higher prevalence in canine CP.[12,13] Canine CP shares many clinical implications with human CP, including the association with life-threatening systemic diseases, such as chronic kidney disease.[14]

Successful reduction or elimination of *P. gingivalis* from the oral cavity is therefore likely to alleviate CP symptoms and progression and may be beneficial in the treatment/prevention of the comorbidities. Despite advances in understanding the CP pathogenesis and incredible progress in the personalized medicine, the treatment has not changed in decades. The protocol for treating periodontitis follows a standardized and straightforward algorithm: (i) review and reinforce oral hygiene; (ii) scaling and root planing; (iii) periodontal surgery if the disease progresses, and (iv) patient enrollment in a periodontal maintenance recall program.[15]

Patient's strict adherence to professional recommendations is pivotal to the success of therapy[16] and subpar maintenance often leads to a relapse of periodontal disease, ultimately causing further tooth loss.

There are several approaches to improve the effect of standard active periodontal therapy.[17] Systemic and topical antibiotics have been shown to improve clinical outcome.[18] However, patients with moderate CP rarely benefit from this adjunctive treatment. Additionally, side effects of the long-term use of antibiotics can be devastating for the patient and can lead to development of antibiotic resistance among various pathogenic species.[19]

Several alternative therapies have been proposed in recent decades. These include: phototherapy, oral statin use, and targeted inhibition *P. gingivalis* virulence factors.[20–22] Herein, we have evaluated the compound bestatin (ubenimex) as a candidate for CP treatment. This bacteria-derived dipeptide produced by *Actinomycetes* spp. is an aminopeptidase N (CD13) inhibitor currently garnering interest as an adjunct cancer therapy. Its beneficial effects have been demonstrated in clinical trials of acute myeloid leukemia or malignant melanoma.[23,24] In eukaryotic cells, bestatin can induce apoptosis, inhibit metastasis, and even sensitize cancerous cells to chemotherapy and radiotherapy, increasing patient survival rates.[25,26] More importantly in the context of this study, bestatin also exhibits antimicrobial properties and was shown to selectively inhibit *P. gingivalis* growth.[27]

Therefore, we evaluated the potential of bestatin as a novel drug to reduce the levels of *Porphyromonas* spp. in planktonic culture, as well as mono- and multispecies biofilms. We also examined the immunomodulatory activity of bestatin in a human neutrophil periodontopathogen infection model, and in a murine model of CP.

2 | MATERIALS AND METHODS

2.1 | Bacteria culture

The following strains were used: *P. gingivalis* W83, *P. gingivalis* ATCC 33277, *P. gulae* ATCC 51700, *Fusobacterium nucleatum* ATCC 25586, *Actinomyces naeslundii* ATCC 12104, *T. forsythia* ATCC 43037, and *Streptococcus gordonii* ATCC 10558. Bacteria were grown in 37°C for 24–72 h before an experiment on BD BBL Brucella agar* with 5% defibrinated sheep blood, 10 μg/mL hemin† and, for optimal growth of *T. forsythia*, 10 μg/mL of N-acetylmuramic acid (NAM).† All, except *S. gordonii*, were grown anaerobically.

* BD, Franklin Lakes, NJ.
† Sigma-Aldrich, St. Louis, MO.

2.2 | Minimum inhibitory concentration assay

Bacteria were grown for 24 h before the experiment in TSB/Y broth[†] with 10 μg/mL hemin, 500 μg/mL L-cysteine,[†] 0.5 μg/mL vitamin K,[†] and 10 μg/mL NAM. OD_{600} (optical density) was measured and adjusted to 0.1. Two hundred microliters of bacteria suspension was transferred to the wells of 96-well plate. Bestatin[†] was added. OD_{600} was measured 24, 48, or 72 h after the start of the experiment, and the results are presented as percentage of the OD_{600} of the untreated wells. Ten microliters of *P. gingivalis* and *P. gulae* suspensions was then plated on blood agar.

2.3 | Monospecies biofilm

Suspensions of *Porphyromonas* spp. were adjusted to OD_{600} = 0.1 in TSB/Y broth and pipetted onto poly-l-lysine[‡] coated 24-well plates. Bestatin was added. After 24 and 48 h of anaerobic incubation planktonic bacteria were gently removed. The adherent bacteria were stained with 0.5% crystal violet[†] for 15 min, wells were then washed twice with H_2O. Residual stain was solubilized in 95% ethanol for 5 min. OD_{595} was measured.

2.4 | Multispecies biofilm

Biofilm of *P. gingivalis* ATCC 33277, *F. nucleatum*, *A. naeslundii*, *T. forsythia*, and *S. gordonii* was established as described previously.[28] Biofilm was allowed to form anaerobically for 48 h (early biofilm, bestatin added at 0 h) or 72 h (established biofilm, bestatin added at 48 h) in anaerobic conditions in 37°C.

2.5 | Polymerase chain reaction

To assess the number of bacteria in biofilm, the quantitative real-time polymerase chain reaction (qRT-PCR) was performed. DNA was extracted from 100 μL of resuspended in 500 μL PBS biofilms with 6% BT Chelex 100 Resin[§] in water according to the manufacturer's instructions. DNA, GoTaq Master Mix,[**] forward and reverse primers were pipetted into wells of a 96-well plate. Primer sequences, reaction protocol, and result analysis were based on the relevant literature.[28]

[‡] Sigma-Aldrich, St. Louis, MO.
[§] Bio-Rad, Hercules, CA.
[**] Promega, Madison, WI.

2.6 | Confocal microscopy

Medium was removed, the early biofilm was fixed with 4% formaldehyde in 4°C for 5 h and dehydrated overnight. Biofilm was rehydrated in PBS, and then incubated overnight in 4°C in 1% bovine serum albumin (BSA) in PBS. Next, 1:5000 DAPI (stock: 5 mg/mL)[††] solution in PBS was added. Biofilm was washed four times and images were acquired via confocal microscopy. Three-dimensional (3D) models were reconstructed from the scanned z-stacks of 5.98 μm thickness (49 slices per z-stack).

2.7 | Neutrophil isolation

Samples of peripheral blood were purchased from the Blood Bank of Haukeland University Hospital, Bergen, Norway. Samples were donated by healthy volunteers anonymously, and are not identifiable. As such they are exempt from need for ethical approval (rekportalen.no/#hjem/s%C3%B8ke_REK). Blood was collected into spray-dried K2EDTA vacutainers[‡‡] and layered 1:1 onto Polymorphprep[§§] and centrifuged at 500 g, RT, for 30 min. Second buffy coat was collected into a fresh tube containing PBS. Cells were centrifuged at 280 g, RT for 10 min. Supernatant was removed, 2 mL of water were added, and cells were incubated for 30 s to lyse residual erythrocytes. Next, 2 mL of 2x concentrated PBS were added, and cells were centrifuged at 280 g, RT for 10 min. Pellet containing >90% neutrophils was suspended in PBS. Cells were stained with 0.2% Trypan Blue and counted.

2.8 | Bacteria staining

Bacteria were stained with pHrodo Deep Red Mammalian and Bacterial Cell Labeling Kit[***] according to the manufacturer's instruction. Stained bacteria were suspended in PBS at a required optical density and used to evaluate the rate of phagocytosis.

2.9 | Phagocytosis

Fifty microliter of neutrophil suspension (8 mln/mL in HBSS (BD, Franklin Lakes, New Jersey)[§]) was transferred to a black 96-well plate. Fifty microliter of HBSS, 5 μg/mL

[††] Sigma-Aldrich, St. Louis, MO.
[‡‡] Greiner Bio-One, Kremsmunster, Austria.
[§§] Axis-shield, Dundee, UK.
[***] ThermoFisher, Waltham, MA.

bestatin and/or 20 μg/mL cytochalasin D[†] were added. Cells were incubated for 30 min at 37°C. Then, pHrodo deep, red-stained bacteria were added (multiplicity of infection (MOI) of 20 bacteria per one ekuaryotic cell) and incubated for 2 h in 37°C. A 100 μL of PBS was added, and phagocytosis was evaluated via flow cytometry and analyzed in FlowJo software.[†††]

2.10 | Reactive oxygen species (ROS) production

Twenty-five microliter of the neutrophil suspension (2 mln/mL in HBSS) was transferred to a white 96-well plate. Twenty-five microliter of HBSS or 5 μg/mL bestatin were added, and cells were incubated for 30 min at 37°C. Then 25 μL of HBSS, bacteria suspension (MOI of 5), or 3 μM n-formylmethionine-leucyl-phenylalanine (fMLP)[†] were added to the cells, followed by 75 μL of 134 μM luminol[†] solution in HBSS. Luminescence was measured every minute for 60 min at 37°C.

2.11 | Human neutrophil elastase (HNE) release

Fifty microliter of neutrophil suspension (6 mln/mL in HBSS) was added to a tube. Fifty microliter of HBSS or 5 μg/mL bestatin were added, and cells were incubated for 30 min at 37°C. Then, 50 μL of HBSS, bacteria suspension (MOI of 5), or 15 μg/mL phorbom 12-myristate 13-acetate (PMA) were added. Samples were incubated for 45 min in 37°C. Cells were centrifuged at 300 g, 10 min, RT, and 100 μL of supernatant was pipetted onto a transparent 96-well plate. Hundred microliter of N-methoxysuccinyl-AAPV-pNA[†] suspended in 100 mM Tris, pH 7.5, 5 mM $CaCl_2$ was added to a final concentration of 50 μM. Absorbance gain at 405 nm was measured every 30 s for 60 min at 37°C.

2.11.1 | Murine experimental periodontitis and bestatin treatment

Specific pathogen-free female Balb/c mice, 12-weeks old, were purchased from Janvier Labs.[‡‡‡] Mice were housed in positively ventilated cages, and fed a standard laboratory diet. Control and bacteria-infected mice ($n = 7$) were housed in separate cages. Animal procedures were performed in accordance with the protocols laid by the Institutional Animal Care and Use I Regional Ethics Committee on Animal Experimentation, Kraków, Poland (Decision No. 362/2020). Experimental periodontitis in mice was induced by oral infection of periodontal tissues with 10^9 CFU of *P. gingivalis* W83 in 100 μL of 2% methylcellulose.[§§§] Each mouse was infected every second day over the course of 3 weeks. Mice in the control group were administered methylcellulose alone. From the last day of infection and for the following 28 days, two groups of mice were treated with in-feed bestatin at two doses: 80 or 400 mg/kg body weight per day. The two control groups (untreated and *P. gingivalis*-infected group) were provided with bestatin-free feed. The procedure was repeated in three independent experiments.

2.12 | Alveolar bone loss

Mice were scanned twice: baseline measurement (T_0) and after bestatin treatment (T_{end}). Anesthetized mice were scanned using high resolution animal microcomputed tomography (micro-CT)[****] to determine changes in bone volume in cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) length in the jaw. Imaging was performed at an ultra-focus magnification, 50 kV source voltage and 0.21 mA current. Three-dimensional images were obtained using the PMOD software,[††††] and a linear distance from the CEJ to the ABC of each tooth of the lower and upper jaw was measured. Each measurement was performed three times.

2.13 | Myeloperoxidase (MPO) assay

The gingival tissues were surgically excised with a scalpel, homogenized in hexadecyl trimethyl ammonium bromide and dissolved in potassium phosphate. MPO activity was measured with a commercial kit.[‡‡‡‡]

2.14 | Statistical analysis

Each experiment was performed at least three times in duplicate. Statistical analysis was done via GraphPad Prism 9. Statistical significance was evaluated with one-way ANOVA with post-hoc test. Results were considered significant when $p < 0.05$.

[†††] BD, Franklin Lakes, NJ.
[‡‡‡] Le Genest-Saint-Isle, France.
[§§§] Sigma-Aldrich, St. Louis, MO.
[****] MILabs, Houten, Netherlands.
[††††] PMOD, Zurich, Switzerland.
[‡‡‡‡] Sigma-Aldrich, St. Louis, MO.

3 | RESULTS

3.1 | Bestatin has bacteriostatic activity against *Porphyromonas* spp

Previous studies demonstrated that bestatin has bacteriostatic, but not bactericidal, activity against *P. gingivalis*.[27] We confirmed bacteriostatic activity against *P. gingivalis* and subsequently evaluated its activity against *P. gulae*, the major periodontal pathogen in dogs, using a microplate assay. Bestatin showed higher efficacy against *P. gulae* than *P. gingivalis* (Figure 1A, B) and significantly restricted bacterial growth at concentrations as low as 0.25 μg/mL for *P. gingivalis* and 0.1 μg/mL for *P. gulae*. Complete growth inhibition was observed when both species were exposed to 2.5 μg/mL bestatin. Interestingly, bestatin was bactericidal against *P. gulae* at concentrations >75 μg/mL, but not against *P. gingivalis*.

Expanding this analysis to other oral bacteria, susceptibility to bestatin was restricted to *Porphyromonas* spp. and the compound showed no activity against *T. forsythia*, *A. naeslundii*, or *S. gordonii*. Only *F. nucleatum* growth was partially inhibited at higher concentrations (Figure 1D).

3.2 | Bestatin inhibits the formation of monospecies biofilms

We next assayed the formation of monospecies biofilms of *P. gingivalis* ATCC 33277, W83 and *P. gulae* ATCC 51700 in the presence of bestatin using crystal violet staining to quantify biofilm biomass. At concentrations of 1 and 5 μg/mL, bestatin prevented the formation of biofilms of all three strains examined, with the highest efficiency observed against *P. gingivalis* ATCC 33277, which demonstrated an 80% reduction at the lowest bestatin dose (Figure 1C). However, pre-established biofilm was resistant to bestatin treatment at 1 or 5 μg/mL (data not shown).

3.3 | Bestatin affects the species composition of multispecies biofilms

To better mimic the oral biofilm formation on the teeth surface, a five species model was used. Bacteria were cocultured, and bestatin was added to the medium either before biofilm formation (early biofilm) or after biofilm was established. An overall reduction in bacteria counts was noted in all tested conditions after bestatin treatment (Figures 2A and 3A), but the most striking differences were observed when biofilm was formed in the presence of bestatin (time 0 h).

The presence of bestatin eliminated 99% of *P. gingivalis* in an early biofilm at all tested concentrations (Figure 2B), and altered the growth of *F. nucleatum* (Figure 2D). Interestingly, we observed a decrease in numbers of the red complex species *T. forsythia* (Figure 2E) following exposure to bestatin, despite no observable effect in the microplate assay (Figure 1D). Loss of *P. gingivalis* from the biofilm was associated with increased numbers of *S. gordonii* (three-fold at 31 μg/mL and two-fold at 155 μg/mL) (Figure 2C). The growth and frequency of *A. naeslundii* in the treated biofilm was unaffected (Figure 2F).

Unsurprisingly, the effect of bestatin on the composition of a pre-established, 48-h biofilm was much less pronounced (Figure 3A) than treatment at time 0 h (Figure 2A). At this time point, only *P. gingivalis* numbers were significantly reduced by bestatin. Even the lowest tested concentration (31 μg/mL) provided a significant reduction of *P. gingivalis* CFUs of around 50% (Figure 3B). In contrast to the early biofilm model, *S. gordonii* did not dominate the established biofilm following treatment, and only a slight increase in the number of CFU was observed (Figure 3C). *F. nucleatum*, *T. forsythia*, and *A. naeslundii* counts remained largely unchanged regardless of the bestatin concentration (Figure 3D–F).

3.4 | Bestatin affects the 3D structure of biofilm

We observed that, in addition to *P. gingivalis, F. nucleatum* counts were altered by bestatin treatment. As these two bacteria species serve as a support for biofilm formation, we have evaluated the 3D structures via confocal microscopy. The treated biofilm structure appeared less thick, with frequent gaps throughout (Figure 4A). The species composition and their spatial arrangement were also significantly affected, as the treated biofilm was more abundant with cocci and long rods, when compared with the untreated control where many short rod-aggregates were observed (Figure 4B).

3.5 | Bestatin promotes phagocytosis of *Porphyromonas* spp. by human neutrophils

Bestatin was previously shown to promote the phagocytic functions of neutrophils against yeast and *Escherichia coli*.[29,30] We therefore examined whether bestatin altered neutrophil phagocytosis of the periodontopathogens *Porphyromonas* spp. We observed that neutrophils isolated from peripheral blood of healthy donors were able to

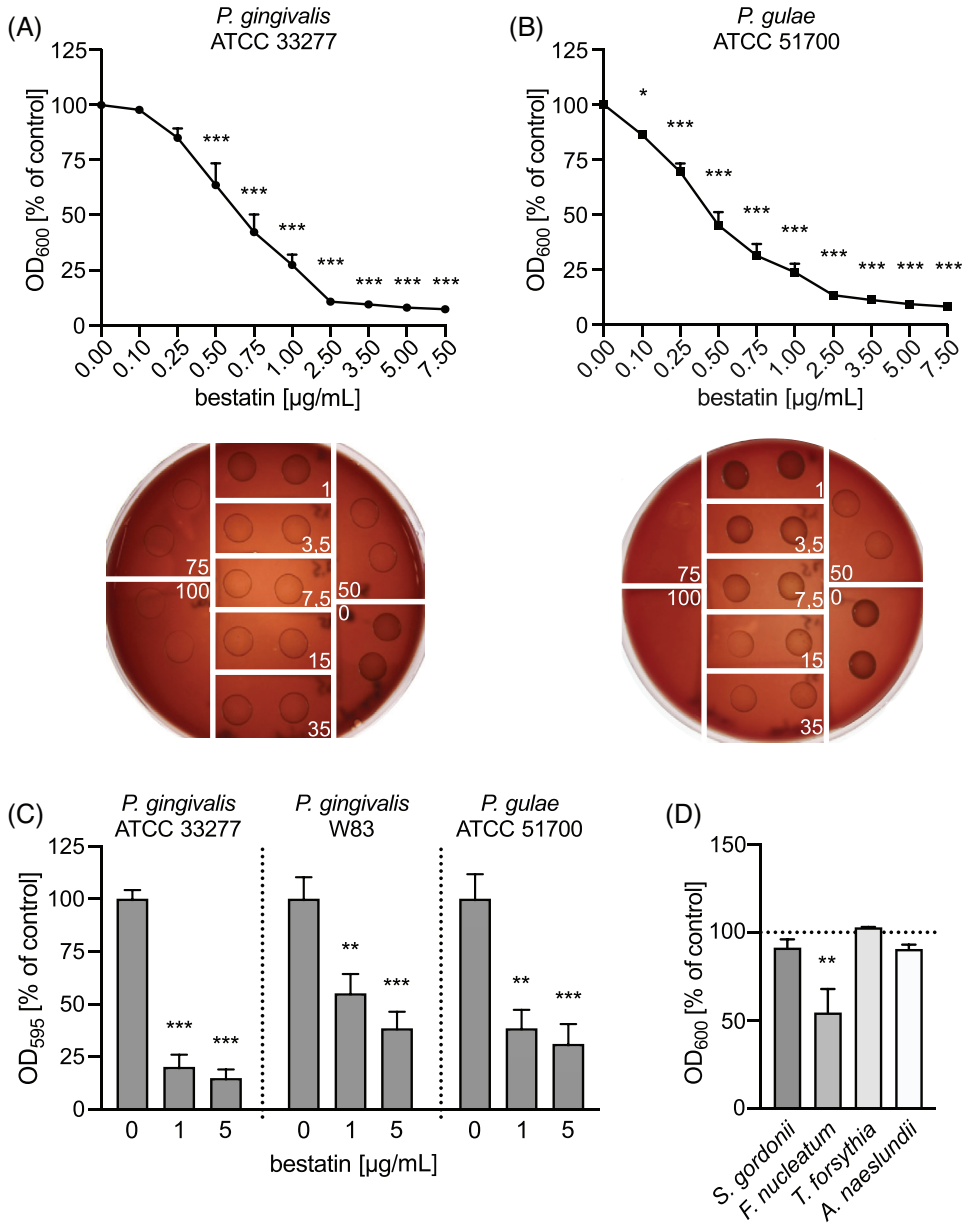

FIGURE 1 Bestatin inhibits *Porphyromonas gingivalis* and *Porphyromonas gulae* growth and biofilm formation, and is partially bacteriostatic against *Fusobacterium nucleatum*, but not other oral bacteria. The bacteriostatic activity of bestatin was evaluated using a microplate assay with *P. gingivalis* ATCC 33277 (A) and *P. gulae* ATCC 51700 (B). After optical density (OD) measurements, 10 μL of the suspension treated with indicated concentrations of bestatin was plated on blood agar plates and grown in anaerobic conditions (representative images of the agar plates, A, B). Monospecies biofilms formed on a poly-l-lysine-coated plate in the presence of indicated concentration of bestatin at 0 h were stained with crystal violet. Stained biofilms were solubilized in 95% ethanol and OD_{595} was measured (C). *Streptococcus gordonii*, *F. nucleatum*, *Tannerella forsythia*, and *Actinomyces naeslundii* were grown in liquid medium in the presence of 300 μg/mL of bestatin and the OD_{600} was measured after 24 or 72 h (*Tannerella forsythia*). Results are expressed as the percentage of the OD measured for the control sample. Each experiment was repeated three times and is shown as means ± SEM. Statistical significance was calculated using a one-way ANOVA with Dunnett post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

phagocytize bacteria more efficiently after a short pre-stimulation with bestatin. The bestatin-treated phagocytes were about 30% (*P. gingivalis* ATCC 33277, Figure 5B; and W83, Figure 5C) or 25% (*P. gulae*, Figure 5D) more effective in bacterial uptake than nontreated cells.

3.6 | Bestatin promotes formation of ROS

ROS play a pivotal role in the function of neutrophils by mediating antibacterial activity, inflammation, and the induction of apoptosis.[31] We found that bestatin

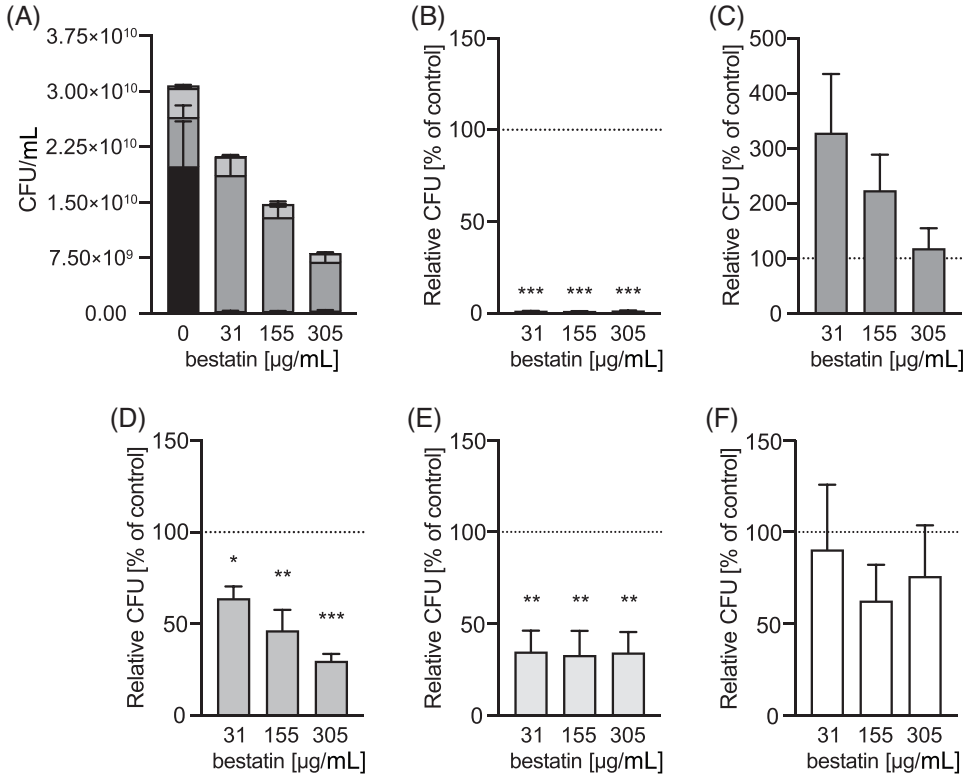

FIGURE 2 Bestatin selectively inhibits growth of *Porphyromonas gingivalis* in early multispecies oral biofilm which alters overall bacterial composition. A mixture of *P. gingivalis* (black), *Streptococcus gordonii* (dark gray), *Fusobacterium nucleatum* (medium gray), *Tannerella forsythia* (light gray), and *Actinomyces naeslundii* (white) was seeded on a poly-l-lysine precoated plate. Bestatin was added at 0 h at the indicated concentrations. At 48 h after the addition of bestatin, medium was removed, and bacterial DNA was extracted via Chelex resin. Bacteria colony forming units (CFUs) were quantified via qPCR (A). The CFUs of *P. gingivalis* (B), *S. gordonii* (C), *F. nucleatum* (D), *T. forsythia* (E), and *A. naeslundii* (F) were compared with the CFUs of the untreated biofilm control in each experiment and expressed as a percentage of the control. Experiments were performed three times and shown as means ± SEM. Statistical significance was determined using a one-way ANOVA and Dunnett post-hoc test. CFU, colony forming units. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

pretreatment increased ROS generation in neutrophils by 50% (Figure 5F). Similarly, neutrophils prestimulated with bestatin generated about 30%–50% more ROS during while exposed to periodontal pathogens than nonstimulated cells. We also examined other bacteria killing mechanisms of neutrophils; however, neither HNE (Figure 5E) nor MPO release (data not shown) were affected by the presence of bestatin.

3.7 | Bestatin protects against bone loss

Next, we assessed the therapeutic efficacy of bestatin in a murine model of CP, by providing bestatin in the animal feed. Since the loss of alveolar bone is a hallmark of CP progression leading to tooth loss in humans and dogs, we assessed this parameter in mice treated with bestatin after oral infection with *P. gingivalis*. Jaws were scanned at the start (T_0) and end of the experiment (T_{END}), and the alveolar bone height was determined. Even a low dose of bestatin (80 mg/kg/daily) protected mice from bone loss in the upper (Figure 6A) and lower (Figure 6B) jaw induced by *P. gingivalis* infection.

Gingival MPO activity was determined as a surrogate measure of neutrophil infiltration of the gingival tissue. When compared with the control group, the *P. gingivalis*-infected group showed a two-fold increase in MPO activity of the gingival tissues (Figure 6C). Bestatin treatment alleviated inflammation of the gingiva in a dose-dependent manner as evident from a significant reduction in MPO activity.

4 | DISCUSSION

Despite advances in understanding the pathogenesis of periodontitis, and incredible progress in the personalized medicine, the treatment of periodontitis has remained unaltered for decades. The global prevalence of this disease has likewise remained high which generates

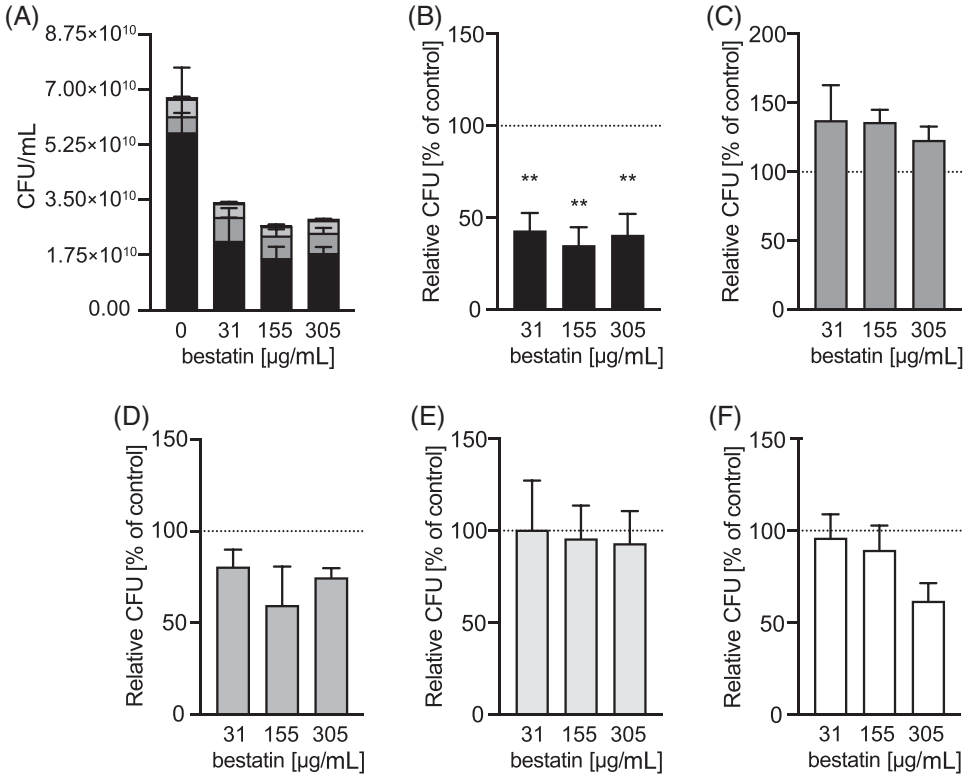

FIGURE 3 Bestatin selectively inhibits growth of *Porphyromonas gingivalis* in pre-established multispecies oral biofilm. A mixture of *P. gingivalis* (black), *Streptococcus gordonii* (dark grey), *Fusobacterium nucleatum* (medium gray), *Tannerella forsythia* (light gray), and *Actinomyces naeslundii* (white) was seeded on a poly-l-lysine precoated plate. Bestatin was added at 48 h at the indicated concentrations. At 24 h after the addition of bestatin, medium was removed, and bacterial DNA was extracted. Bacteria colony forming units (CFUs) were quantified via qPCR (A). The CFUs of *P. gingivalis* (B), *S. gordonii* (C), *F. nucleatum* (D), *T. forsythia* (E), and *A. naeslundii* (F) were compared with the CFUs of the untreated biofilm control in each experiment and expressed as a percentage of the control. Experiments were performed three times and shown as means ± SEM. Statistical significance was determined using a one-way ANOVA and Dunnett post-hoc test. CFU, colony forming units. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

significant societal and economical costs.[11] These costs are increased when also considering CP-associated diseases. Therefore, improving the standard of care, and implementing new prevention/treatment strategies would be highly beneficial.

Herein, we characterize a potential CP drug candidate, bestatin. A study in 1992 first demonstrated its efficacy against several strains of *P. gingivalis*.[32] Since then, this compound was shown to be highly specific towards *P. gingivalis* strains, while ineffective against other oral bacterial species.[33] Indeed, our study confirms that the effects of bestatin are dose-dependently specific to *P. gingivalis* and the established canine periodontopathogen *P. gulae*. While bestatin exhibits bacteriostatic activity against *P. gingivalis*, we also observed bactericidal effects against *P. gulae*. Previous work has suggested that the selectiveness of bestatin stems from its ability to inhibit *Porphyromonas* spp. peptide uptake.[34] Curiously, while bestatin's predominant activity in humans is the inhibition of several aminopeptidases, no such activity was observed against bacterial peptidases.[35]

Except for *F. nucleatum* ATCC 25586, we have confirmed that bestatin had no inhibitory activity on the oral microbiota constituents of our multispecies biofilm model: *A naeslundii* ATCC 12104, *T. forsythia* ATCC 43037, and *S. gordonii* ATCC 10558. These data are consistent with previous reports, where different strains of these species were evaluated.[27] Eradication of *P. gingivalis* from sites of infection, or inhibition of its virulence factors were shown to alleviate symptoms, and even prevent the onset of CP. Indeed, monoclonal antibodies targeting the hemagglutinin 2 domains of gingipains and a Kgp-specific inhibitor were recently shown to protect mice against alveolar bone loss. Kgp-specific inhibitor also reduced neuroinflammation in a murine Alzheimer model.[9,36] Therefore, the selectiveness and efficacy profile of bestatin make it an excellent drug candidate.

Any antimicrobial candidate compound considered for CP treatment must be effective against bacteria residing within biofilms of the dental plaque. These multilayered aggregates embedded in the extracellular matrix significantly increase microbial resistance to antimicrobial

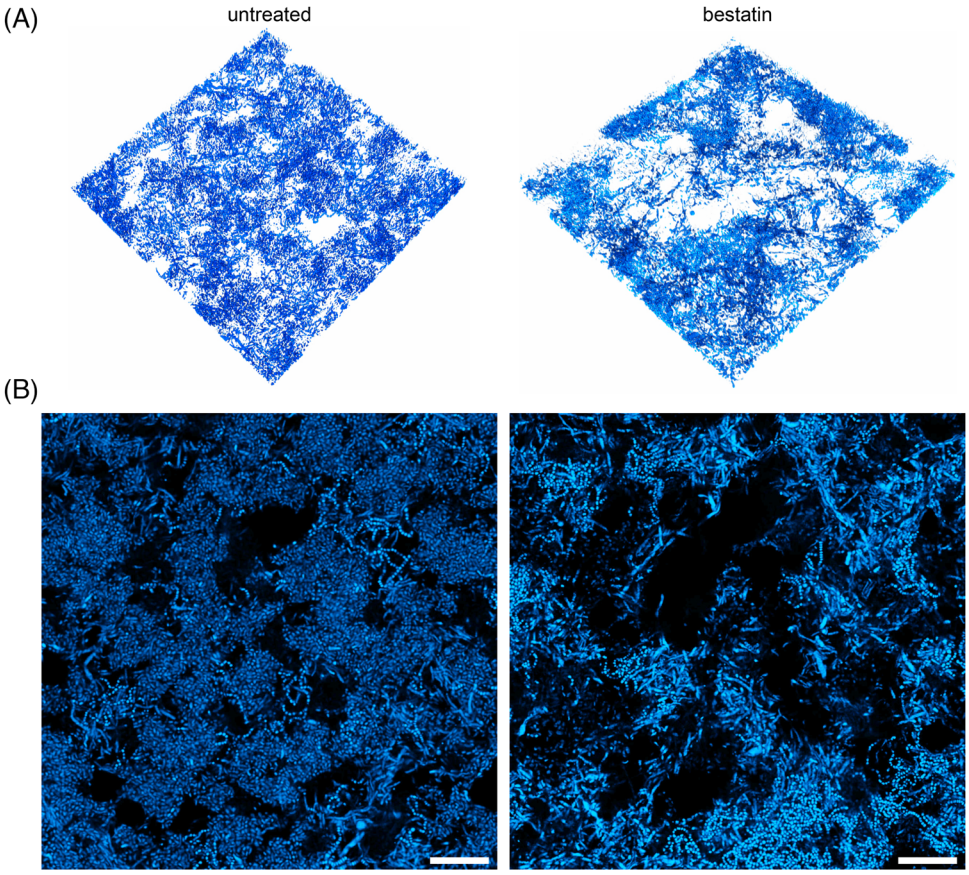

FIGURE 4 Treatment with bestatin significantly affects the structure of the forming multispecies biofilm. A mixture of *Porphyromonas gingivalis*, *Streptococcus gordonii*, *Fusobacterium nucleatum*, *Tannerella forsythia*, and *Actinomyces naeslundii* was seeded on dishes with glass inserts and bestatin/DMSO were added at 0 h. Biofilm was established for 48 h, fixed in 4% paraformaldehyde and dehydrated overnight. Biofilm was rehydrated with PBS, blocked with 1% bovine serum albumin (BSA) in PBS and stained with DAPI. Z-stack images of $77.53 \times 77.53 \times 5.98\ \mu$m were collected using a confocal microscope with the 100× magnification objective, and then three-dimensional reconstruction was performed (A). Representative slices (B) from $z = 3.48\ \mu$m are shown. Scale bar = 10 μm.

agents. Previous reports have indicated that resistance to antimicrobials is increased by as much as 1000-fold in biofilms.[37] For example, *Pseudomonas aeruginosa* grown in biofilms were able to withstand a concentration of tobramycin 50 times higher than the planktonic bacteria.[38] Similarly, we observed that while 1 and 5 μg/mL of bestatin were highly effective against planktonic bacteria, such doses proved unsuccessful against pre-established, 48-h biofilm. Moreover, treatment of biofilms in vivo presents an even bigger challenge due to the synergistic interactions of species within these structures, supporting survival in hostile microenvironments and promoting antibiotic resistance.[39] To mimic the pathogenic biofilm of CP, we tested bestatin in a multispecies oral biofilm model. *P. gingivalis* resistance to bestatin increased ≈ 10–15 times while in biofilm, but this compound was still effective in sub-millimolar concentrations against pre-established biofilm. Use of a compound that targets the keystone CP pathogen in a highly selective manner has many potential benefits for patients. Firstly, it reverses the pathogenic microflora shift to the commensal state, preventing the main trigger of the inflammatory response, and reduces the overall bacterial load. Secondly, this compound eliminates the need for nonspecific, broad-spectrum antibiotics, reducing antibiotic resistance development and the subsequent side effects (e.g., reduction in gut microflora diversity).[40] No significant adverse effects of long-term (up to 13 years) bestatin use were observed in human subjects, highlighting a favorable safety profile of this drug.[41] Importantly, to our knowledge, no report has documented the ability of *Porphyromonas* spp. to develop bestatin resistance, and we observed no resistance development in our study.

The direct antibacterial activity of bestatin will synergize with its immunomodulatory activities, promoting efficient bacterial clearance and quicker resolution of inflammation. Neutrophils are a major component of the immediate host defense against bacterial and fungal infections. In fact, neutrophil deficiency, or impairment of their function results in the early onset of severe periodontitis, for example, exacerbated CP associated with Papillon-Lefevre

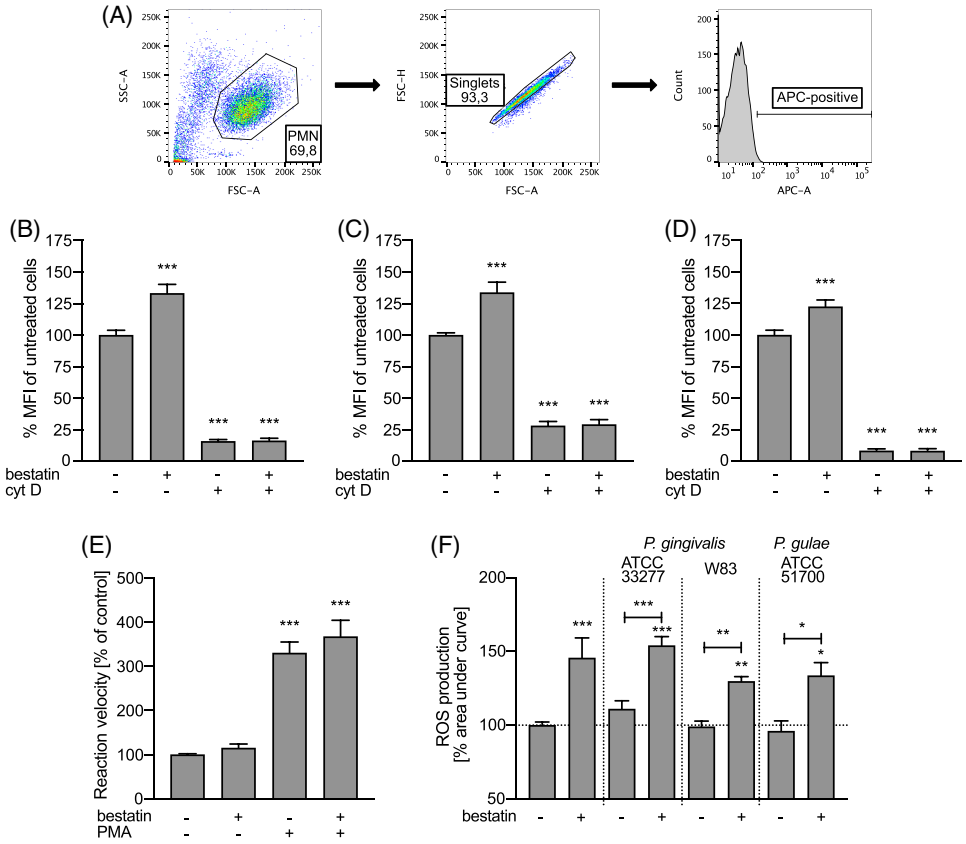

FIGURE 5 Bestatin promotes phagocytosis of bacteria, and generation of reactive oxygen species (ROS), but not the release of human neutrophil elastase (HNE). Peripheral blood was collected from healthy volunteers and neutrophils were isolated. Cells were preincubated with bestatin or cytochalasin D for 30 min at 37°C, and pHrodo deep red-stained *P. gingivalis* ATCC 33277 (B), W83 (C), and *P. gulae* ATCC 51700 (D) were added at a MOI of 20 to the cells for 2 h at 37°C. Cells were analyzed by flow cytometry. pHrodo deep red signal was analyzed using the APC channel. (A) Representative gating strategy used in the analysis of all experiments. Results are expressed as a percentage mean fluorescence intensity of the untreated cells. To evaluate HNE release (E), after preincubation with bestatin, cells were stimulated with PMA, and supernatants were collected after 40 min of incubation at 37°C. HNE activity was determined using an HNE-specific chromogenic substrate. To analyze ROS generation, after preincubation with bestatin, fMLP or bacteria (MOI of 5) and luminol substrate (final concentration: 67 μM) were added and luminescence intensity was recorded for 60 min. Results are presented as the percentage of the area under the curve of the control cells. Experiments were performed at least three times in duplicates and shown as means ± SEM. Statistical significance was determined using a one-way ANOVA with Dunnett (B–E) and Bonferroni post-test (E–F). MFI, mean fluorescence intensity; ROS, reactive oxygen species; MOI - multiplicity of infection; FSC - forward scatter; SSC - side scatter; -A/H - area/height; APC - allophycocyanin; PMA - phorbol 12-myristate 13-acetate; fMLP - n-formymethionine-leucyl-phenylalanine. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

syndrome.[42] Uncontrolled activation of these professional phagocytes contributes to tissue damage and subsequently bone loss in patients suffering from CP, as neutrophils release multiple proteases responsible for extracellular matrix (ECM) degradation, as well as proinflammatory mediators.[43] The inflammatory response in the gums can be curbed through efficient pathogen elimination. Indeed, bestatin appears to promote both macrophage and neutrophil phagocytosis, as was previously demonstrated with fluorescently labeled *E. coli*, as well as yeast particles.[29,30] We have also shown that bestatin induces neutrophil-derived ROS generation. Free radicals are essential for bacteria killing, cell differentiation, autophagy, and apoptosis. Indeed, sensitization of cancer cells to chemo- or radiotherapy is largely attributed to enhanced apoptosis of cancerous cells.[44] However, ROS generation can also contribute to tissue damage. This was most recently described in the case of COVID-19, where overabundant ROS promoted sudden patient decline.[45] *P. gingivalis* has been described to not only escape ROS-driven killing, but to use ROS-derived host tissue destruction to enhance its survival.[46] Subsequently, nanoparticles that scavenge free radicals represent an exciting treatment strategy for CP, preventing oxidative tissue damage.[47] That therapy, however, does not address the underlying cause of the disease—oral dysbiosis. Using a murine model of CP, we showed that bestatin treatment has uniformly beneficial effects to infected mice. Daily administration of bestatin

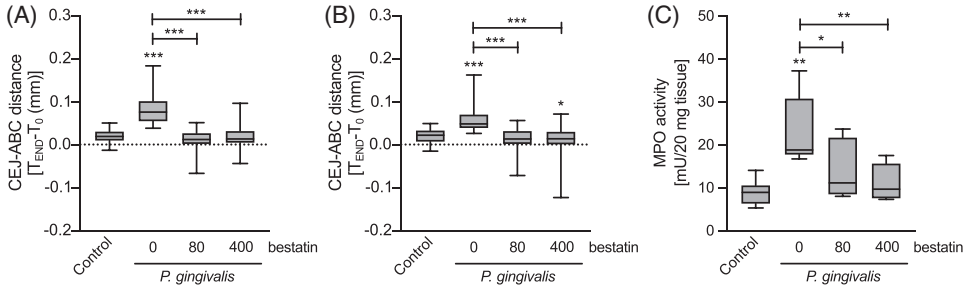

FIGURE 6 Bestatin protects mice from alveolar bone loss over the course of *Porphyromonas gingivalis* infection. Mice ($n = 7$ per group) were scanned at T_0 by microcomputed tomography. Mice were then orally infected with *P. gingivalis* every second day for 3 weeks (with the exclusion of the control group). Groups were subsequently provided with feed ad libitum containing two doses of bestatin (80 and 400 mg/day/kg of body weight) for 4 weeks, while group 0 and the control group were provided with feed without bestatin. At 4 weeks, mice were scanned again and sacrificed. Jaw scans were analyzed and the cemento-enamel junction-alveolar bone crest distance was measured at both timepoints. Bone loss was calculated by subtracting distance at T_0 from distance at the end of the experiment for the upper (A) and lower (B) jaw of each mouse. Gingival tissues were extracted, homogenized and evaluated for myeloperoxidase activity (C). Results are shown as means ± SD (A, B) or SEM (C). Statistical significance was calculated with one-way ANOVA with Bonferroni post-hoc test. CEJ, cemento-enamel junction-alveolar bone crest; MPO, myeloperoxidase. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

through the animal feed efficiently prevented alveolar bone resorption, and caused no adverse effects to the treated animals. Moreover, markedly lower levels of MPO activity detected in the gingival tissue clearly indicated that the compound enabled better control of local inflammation. This treatment outcome is comparable to those observed upon gingipain inhibition or infection with a gingipain-null strain,[48] but is achieved through the control of bacterial growth and stimulation of the immune system, promoting rapid bacterial clearance and preventing local chronic inflammation.[35]

Bestatin presents an excellent treatment candidate for canine periodontitis—a disease caused predominantly by *P. gingivalis* and *P. gulae*.[12,13] Despite often rigorous care, some studies estimate the prevalence of CP in dogs to be as high as 100%, affecting animals as young as 1 year old.[49] We demonstrate that *P. gulae* is more susceptible to bestatin treatment than *P. gingivalis*. Furthermore, to our knowledge, this study provides the first evidence that food supplementation with this compound is a sufficient CP preventive measure, which could reduce the need for specialized veterinary interventions.

5 | CONCLUSIONS

This study shows that bestatin is a promising candidate for a clinical trial. It is efficient in selectively controlling periodontopathogen proliferation, stimulating phagocytosis, and preventing local tissue destruction. More importantly, oral administration of the drug via the animal feed was sufficient to improve clinical outcome, suggesting that addition of bestatin to tooth paste or mouthwash would be an adequate method to reduce CP prevalence in the human population.

AUTHOR CONTRIBUTIONS

Study design: Jan Potempa and Piotr Mydel; data acquisition: Marta Kaminska, Malgorzata Benedyk-Machaczka, Karina Adamowicz, Ardita Aliko, Kamila Drzazga, Klaudia Słysz, Ewa Bielecka; analysis: Marta Kaminska, Malgorzata Benedyk-Machaczka; manuscript draft: Marta Kaminska; manuscript revision: Marta Kaminska, Malgorzata Benedyk-Machaczka, Karina Adamowicz, Ardita Aliko, Kamila Drzazga, Klaudia Słysz, Ewa Bielecka, Jan Potempa, and Piotr Mydel. All authors have read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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