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RESEARCH ARTICLE

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Targeted elimination of *Fusobacterium nucleatum* alleviates periodontitis

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ABSTRACT

Background: *Fusobacterium nucleatum*, a pathobiont in periodontal disease, contributes to alveolar bone destruction. We assessed the efficacy of a new targeted antimicrobial, FP-100, in eradicating *F. nucleatum* from the oral microbial community *in vitro* and *in vivo* and evaluated its effectiveness in reducing bone loss in a mouse periodontitis model.

Methods: A multispecies bacterial community was cultured and treated with two concentrations of FP-100 over two days. Microbial profiles were examined at 24-h intervals using 16S rRNA sequencing. A ligature-induced periodontitis mouse model was employed to test FP-100 in vivo.

Results: FP-100 significantly reduced *Fusobacterium spp*. within the in vitro community ($p < 0.05$) without altering microbial diversity at a 2 μM concentration. In mice, cultivable *F. nucleatum* was undetectable in FP-100-treated ligatures but persistent in controls. Beta diversity plots showed distinct microbial structures between treated and control mice. Alveolar bone loss was significantly reduced in the FP-100 group ($p = 0.018$), with concurrent decreases in gingival IL-1β and TNF-α expression ($p = 0.052$ and 0.018, respectively).

Conclusion: FP-100 effectively eliminates *F. nucleatum* from oral microbiota and significantly reduces bone loss in a mouse periodontitis model, demonstrating its potential as a targeted therapeutic agent for periodontal disease.

KEY MESSAGES

FP-100 eliminates *F. nucleatum* from an *in vitro* multispecies microbial community at low doses without affecting bacterial diversity.

FP-100 treatment leads to the *in vivo* elimination of *F. nucleatum*, reducing alveolar bone loss and levels of pro-inflammatory cytokines in the gingiva.

FP-100 is a new antimicrobial to target *F. nucleatum*-mediated periodontal disease.

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Antimicrobial agents; *Fusobacterium nucleatum*; periodontal disease; periodontal bone loss; hygromycin a

Introduction

Antimicrobial resistance is increasing at an alarming rate worldwide [1]. The inappropriate use of broad-spectrum antibiotics has led to the emergence of pan-resistant superbugs [2,3]. Long-term treatments enable antibiotic-resistant species to colonize the spaces vacated by commensal flora, further complicating treatment outcomes. Thus, targeted antimicrobial therapies are needed, which offer promise by selectively inhibiting pathogens of interest without adversely affecting commensal species. These strategies have proven beneficial in various pathological processes, including periodontal diseases [4].

Fusobacterium spp. are opportunistic pathogens commonly found in the oral cavity and gastrointestinal tract, implicated in many oral and systemic diseases. *Fusobacterium nucleatum*, in particular, is prevalent in conditions such as periodontal disease, pulp infection, and oral cancer [5,6]. Discovered as a part of the oral microbiome associated with periodontal diseases, the abundance of this species increases with the severity of periodontal inflammation [7]. *F. nucleatum* is distinguished by various virulent characteristics, particularly its adhesion proteins [5,8]. These proteins promote attachment to host cells and initial colonizing bacteria, thereby serving as a 'bridging organism' within dental plaque. *F. nucleatum*'s role is critical for supporting the adherence and proliferation of other periodontal pathogens by providing mechanical stability and metabolic benefits [8–10]. *F. nucleatum* can also induce the invasion of other pathogens by fostering epithelial–mesenchymal transformation in gingival epithelial cells and manipulating the local immune environment, favoring the progression of periodontal disease [11–13]. We have shown that *F. nucleatum*

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can survive in neutrophils, hampers the oxidative killing mechanism of these cells by inhibiting superoxide production and can use the host cells for dissemination between different organs [12,13]. Systemic dispersion of oral *F. nucleatum* is linked with other health issues, including colorectal cancer, adverse pregnancy outcomes, and, more recently, Alzheimer's disease [14–18]. The targeted elimination of *F. nucleatum* is a justified strategy to treat oral diseases while preventing the systemic pathologies associated with its trafficking to distant organs.

FP-100 is a selective antibiotic (hygromycin A) targeting ribosomes in spirochetes. It was rediscovered while searching for targeted treatments against the Lyme disease pathogen, *Borrelia burgdorferi* [19]. While primarily efficacious against *Treponema* species at minimal doses, FP-100 also inhibits the growth of *F. nucleatum* without harming the gut's commensal bacteria such as *Streptococcus parasanguinis*, *Bifidobacterium longum*, and *Enterococcus faecalis*. Considering the adverse effects of commonly used broad-spectrum antimicrobial agents in adjunctive periodontal therapy, we hypothesized that FP-100 may offer a promising treatment for periodontal disease. Thus, this study aimed to evaluate the impact of FP-100 on the multispecies microbial community associated with periodontitis and to assess its potential to prevent periodontal inflammation and destruction in an *F. nucleatum*-induced periodontitis model in mice.

Materials and methods

Efficacy of FP-100 on a multispecies microbial community

To assess the impact of FP-100 on a human multispecies microbiome, we established an *in vitro* multispecies community from the subgingival plaque of a patient with clinically diagnosed periodontitis. The patients were enrolled at the Center for Clinical and Translational Research under the ADA Forsyth IRB protocol number IRB #16–08 and were provided informed consent before sampling. Subgingival plaque samples were collected using Gracey curettes from a 6 mm pocket depth and quickly suspended in 350 µl of PBS supplemented with 20% glycerol. Fifty microliters of the frozen stock of each subgingival plaque suspension was inoculated into 2 ml pre-reduced SHI medium [20], and the rest of the suspension was stored at −80°C for later use. The cultures were incubated overnight at 37°C in a microaerophilic environment (2% oxygen, 5% carbon dioxide, balanced with nitrogen). The microbial profiles of the resultant communities in the SHI medium were determined by 16S rRNA V1V3 amplicon sequencing. The plaque sample that gave rise to a community with the highest abundance of *Fusobacteria spp*. was chosen to establish an *in vitro* multispecies community and test the efficacy of FP-100 in eliminating *Fusobacteria spp*. Briefly, 50 µl of the frozen stock of the subgingival plaque suspension was inoculated into a 2 ml pre-reduced SHI medium. The culture was incubated at 37°C in a microaerophilic environment. The MIC for FP-100 on *F. nucleatum* is 1 µg/ml [19]. After 24-h incubation, the culture was then diluted at a ratio of 1:10 in fresh SHI medium, and the resultant diluted culture was used to set up 2 treatment groups with different FP-100 concentrations of 1 µg/ml (~2 µM) and 5 µg/ml (~10 µM), respectively. The FP-100 was added to 2 ml of diluted culture for each treatment group to final concentrations of 1 µg/ml and 5 µg/ml. A vehicle-treated control group was also set up. The cultures were then incubated in a microaerophilic environment and passaged at 24-h intervals for 48 h at a 1:10 dilution into a fresh medium containing the respective concentrations of FP-100.

Five hundred microliters of the bacterial culture were collected every 24 h. The cells were harvested by centrifugation at 17,000 × g for 10 min, and genomic DNA was extracted using the MasterPure complete DNA Purification Kit (Epicentre) according to the manufacturer protocol. DNA concentration was determined with the Nanodrop 2000 spectrophotometer (Thermo Scientific), and samples were stored at −80°C until 16S rRNA gene sequencing to profile the bacterial communities.

Sequencing was performed on the Illumina MiSeq platform, and bioinformatic processing was conducted by Zymo Research Corporation (CA, USA) and the ADA Forsyth Oral Microbiome Core. Taxonomic assignment utilized a comprehensive set of 16S rRNA reference sequences, including MOMD v0.1, HOMD v15.2, HOMD 16S rRNA RefSeq Extended v1.1, GreenGenes (GG), and the NCBI 16S rRNA reference sequence collection. The microbial community composition was analyzed using QIIME 2 software, with alpha diversity assessed by the Kruskal–Wallis H test, and ANCOM-BC was employed for the differential abundance analysis.

Mouse periodontitis model

We used a mouse periodontitis model to test the efficacy of targeted elimination of *F. nucleatum in vivo* and prevent *F. nucleatum*-induced periodontal disease. Twenty 8-week-old C57BL/6 mice were randomly assigned to treatment and control (vehicle) groups. The mice were housed in a specific pathogen-free environment, with a 12:12-hour light/dark cycle at 24 ± 0.5°C and 40–70% relative humidity. The Institutional Animal Care and Use Committee (IACUC) of the ADA Forsyth Institute reviewed and approved the experimental protocols.

Overnight cultures of *F. nucleatum* (ATCC 23726) were adjusted to the turbidity of a 0.5 McFarland standard. As previously detailed, silk ligatures immersed in the culture for 1 min were used to induce periodontitis

by application around the maxillary second molars [21]. Starting the day after ligature placement, FP-100 was administered to the test group mice via oral gavage at 200 mg/kg daily, at 3 mg/ml, while the control group received the vehicle (distilled water). Both the antibiotic and vehicle were administered twice daily. One hundred microliters of *F. nucleatum* suspension was administered to both groups in the interval between two antibiotic administrations. One week later, the animals were euthanized. Ligatures were collected for microbial analyses; maxillae were harvested to measure alveolar bone loss, and the keratinized gingiva surrounding the teeth was excised to measure the expression of pro-inflammatory cytokines IL-1β and TNF-α.

Recovery of F. nucleatum from ligatures and microbial profiling of the oral microbiome

The content of the ligature was extracted into 150 µl of PRAS dilution blank (Anaerobe Systems) with 30 min of shaking on ice. Subsequently, 10 µl of this solution was inoculated onto blood agar plates and incubated anaerobically for 48 h to determine the presence of cultivable *F. nucleatum*. After the incubation period, 2 ml of sterile phosphate-buffered saline (PBS) was added to the culture plates, and the colonies were dispersed within the PBS using a sterile loop. The suspension was then collected, and DNA extraction using the boiling method [22] followed by real-time PCR analysis. The *Fusobacterium* adhesion A (FadA) gene, which is the most significant virulence factor identified as highly conserved in oral *Fusobacterium*, was used to quantify *F. nucleatum* [5]. All PCR tests were also validated with 16S rRNA primer. The presence of *F. nucleatum* was confirmed by real-time PCR with SYBR Green and species-specific primers (F: TGCAGCAAGTTTAGTAGGTG; R: CATTGTAAA CTTGTTCATTTTGT) with StepOne™ Real-Time PCR System (Applied Biosystems™). The PCR reaction was conducted using an initial cycle of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s. DNA from the ligature content of five samples from each of the vehicle and FP-100 groups were isolated using the MasterPure™ kit (Biosearch Technologies) and subjected to 16S rRNA sequencing to assess microbial diversity and relative abundance, as detailed above.

Alveolar bone loss assessment

Bone morphometric analysis was used to evaluate the severity of alveolar bone loss among the groups. After euthanasia and ligature removal, the maxillae were subjected to defleshing by dermestid beetles for 4–5 days. Subsequently, the samples were immersed in 5% hydrogen peroxide for 8 h to ensure cleaning, followed by rinsing with water. To enhance contrast, the samples were stained for 10 s using methylene blue (1% solution in water), allowing clear differentiation between bone and dental structures before the morphometric analysis.

The prepared specimens were mounted and imaged from both buccal and palatal sites in the left and right maxillae at 10X magnification with an inverted microscope (Axiovert 200, Zeiss, Thornwood, NY, USA) using AxioVision 4.8 software. Measurements of the area between the alveolar bone crest and the cementoenamel junction for each of the three maxillary molars were performed using ImageJ Software, and the results were expressed in square millimeters.

Analysis of inflammatory gene expression in gingiva

Gingival samples, stored in RNA stabilization solution (RNA*later*, ThermoFisher Scientific) until processing, underwent mRNA extraction using a commercially available kit (RNeasy, Qiagen). From each sample, 1 µg of RNA was converted to cDNA using a reverse transcription kit (Applied Biosystems™). PCR reactions utilized gene-specific oligonucleotide probes for murine IL-1β (Mm00434228_m1, ThermoFisher Scientific) and TNF-α (Mm00443258_m1, ThermoFisher Scientific), with 5 µl of TaqMan Universal Master Mix® and 0.5 µl of the specific probe, adjusted to a final volume of 9 µl with water. The reaction included 1 µl of cDNA and followed these conditions: an incubation step at 95°C for 20 sec, then 40 cycles of 1 sec at 95°C and 20 sec at 60°C using the real-time PCR instrument (StepOnePlus™, Applied Biosystems). Data was evaluated using the comparative ΔΔCt method [23], with mouse GAPDH (435292E, Applied Biosystems™) as the endogenous control. The relative expression of individual samples compared to the mean of the Vehicle group was calculated by 2^-ΔΔCt, reported as fold change.

Statistical analysis

Morphometric analysis data were analyzed using GraphPad Prism Software v9.10. The Shapiro – Wilk test verified the normality of the data distribution. An unpaired t-test compared two groups. Data are presented as mean ± SEM, with $p < 0.05$ denoting significance.

Results

Selective elimination of Fusobacterium species by low-dose FP-100 without significantly affecting microbial diversity

In vitro multispecies community derived from the subgingival plaque of a periodontitis patient exhibited a diverse bacterial population. *Fusobacteria spp*., particularly *F. nucleatum* and *F. periodonticum*, were abundant, constituting over 25% of the microbial

community. While the relative abundance of *Fusobacteria* spp. remained relatively stable over 2 days in the control culture, FP-100 effectively reduced *Fusobacterium spp* at both tested concentrations ($p < 0.05$). With 1 µg/ml (~2 µM) of FP-100, there was a marked reduction in the relative abundance of *Fusobacteria* to 3.5% by day one and 0.08% by day two. At a higher concentration of 5 µg/ml (~10 µM), the reduction was 8.3% on day one and 0.2% by day two.

The lower dose of FP-100 (1 µg/ml) did not significantly alter the alpha diversity of the microbial community compared with the non-treatment control. Although not statistically significant, a higher dose of FP-100 (5 µg/ml) treatment resulted in a more drastic reduction in alpha diversity at day 2 compared to the non-treatment group, as reflected by a more notable decrease in observed species, Shannon and Simpson indices (Figure 1).

Suppression of Fusobacterium growth in ligature-induced periodontitis model by FP-100

F. nucleatum colonies were detectable in every sample from the control group. In contrast, samples from the treatment group showed no *F. nucleatum* colonies (Table 1). 16S rRNA sequencing analysis revealed a decrease in alpha diversity within the oral microbiota of the treatment group; however, this reduction did not result in a statistically significant difference in the mean alpha diversity in observed features, Shannon or Simpson indices ($p > 0.05$). Furthermore, the relative abundance of *F. nucleatum* was reduced to a mean of 3.8% in the treatment group, compared to 16.7% in the control group (adjusted p (q) = 0.001). *Mammalicoccus lentus*, a member of the mouse commensal microenvironment, showed a significantly higher relative abundance in the treatment groups ($q < 0.001$) (Figure 2).

Alleviation of alveolar bone loss and pro-inflammatory cytokine expression through FP-100 treatment

Morphometric analysis showed that FP-100 treatment alleviated alveolar bone loss. Specifically, the average bone loss in the FP-100 group was measured at $0.14 \pm 0.02\ mm^2$, compared to $0.18 \pm 0.03\ mm^2$ in the vehicle group, with a statistically significant difference ($p = 0.016$). Additionally, there was a significant decrease in TNF-α expression within the FP-100 group ($p = 0.018$). Expression of IL-1β was also reduced in the FP-100 treated group compared to the vehicle-treated controls ($p = 0.052$) (Figure 3).

Discussion

Our findings indicated that FP-100 administration effectively eradicated *F. nucleatum in vitro* and *in vivo*, thereby reducing gingival inflammation and alveolar bone loss in a mouse model of ligature and *F. nucleatum*-induced periodontitis. This study was the first to evaluate the efficacy of a targeted antibiotic, FP-100, treatment against oral *F. nucleatum*.

F. nucleatum elicits a robust immune response in periodontal tissues, inducing the expression of inflammatory cytokines from gingival fibroblasts and antimicrobial peptides from epithelial cells [24,25]. Furthermore, we recently showed that *F. nucleatum* can survive within neutrophils, facilitating its dissemination to distant sites of the body [26]. The pathogenicity of *F. nucleatum* is further demonstrated through its interactions with other periodontal pathogens. It enhances the invasiveness of *P. gingivalis* [27–29], and acts synergistically with *Tannerella forsythia* [9,30]. A synergistic relationship was also reported between *F. nucleatum* and *Treponema denticola*. In this interaction, *T. denticola* suppressed the immune responses against *F. nucleatum* [31]. FP-100 was reported to eradicate *T. denticola* strains at low doses, a process facilitated by the uptake of the compound by *Treponema* sp. through their nucleoside transporter [19]. The ability of the compound to effectively disrupt the bidirectional interaction between *T. denticola* and *F. nucleatum* further underscores its potential value in the management of periodontal disease.

Targeting the elimination of *F. nucleatum* is crucial not only for oral health but also to prevent its extraoral impacts. As an oral bacterium, *F. nucleatum* can colonize distant organs and is associated with severe pathologies [32]. It disseminates to non-oral sites via the bloodstream [33]. Our recent research demonstrated that neutrophils may play a crucial role in this dissemination, acting as a 'Trojan horse' for carrying the bacterium to distant sites [26]. Thus, *F. nucleatum* poses a significant risk for oral-systemic disease connection, and its targeted elimination is critical to disrupting this link to prevent systemic complications associated with oral diseases. To this end, *F. nucleatum* has been identified in tumor tissue in colorectal cancer, with its abundance linked to increased tumor growth and a poorer prognosis [34]. Antibiotic treatment targeting *F. nucleatum* has been correlated with reduced tumor growth [35]. Additionally, periodontal therapy has decreased fecal levels of *F. nucleatum* [36]. Therefore, therapies designed to eradicate oral *Fusobacteria* would not only treat intraoral conditions, such as periodontitis and halitosis [37,38] but also reduce the risk of systemic diseases such as colorectal cancer [35].

Emerging therapeutic approaches against this bacterium cover vaccines, plant extracts, probiotics, bacteriophages, and engineered bioactive molecules,

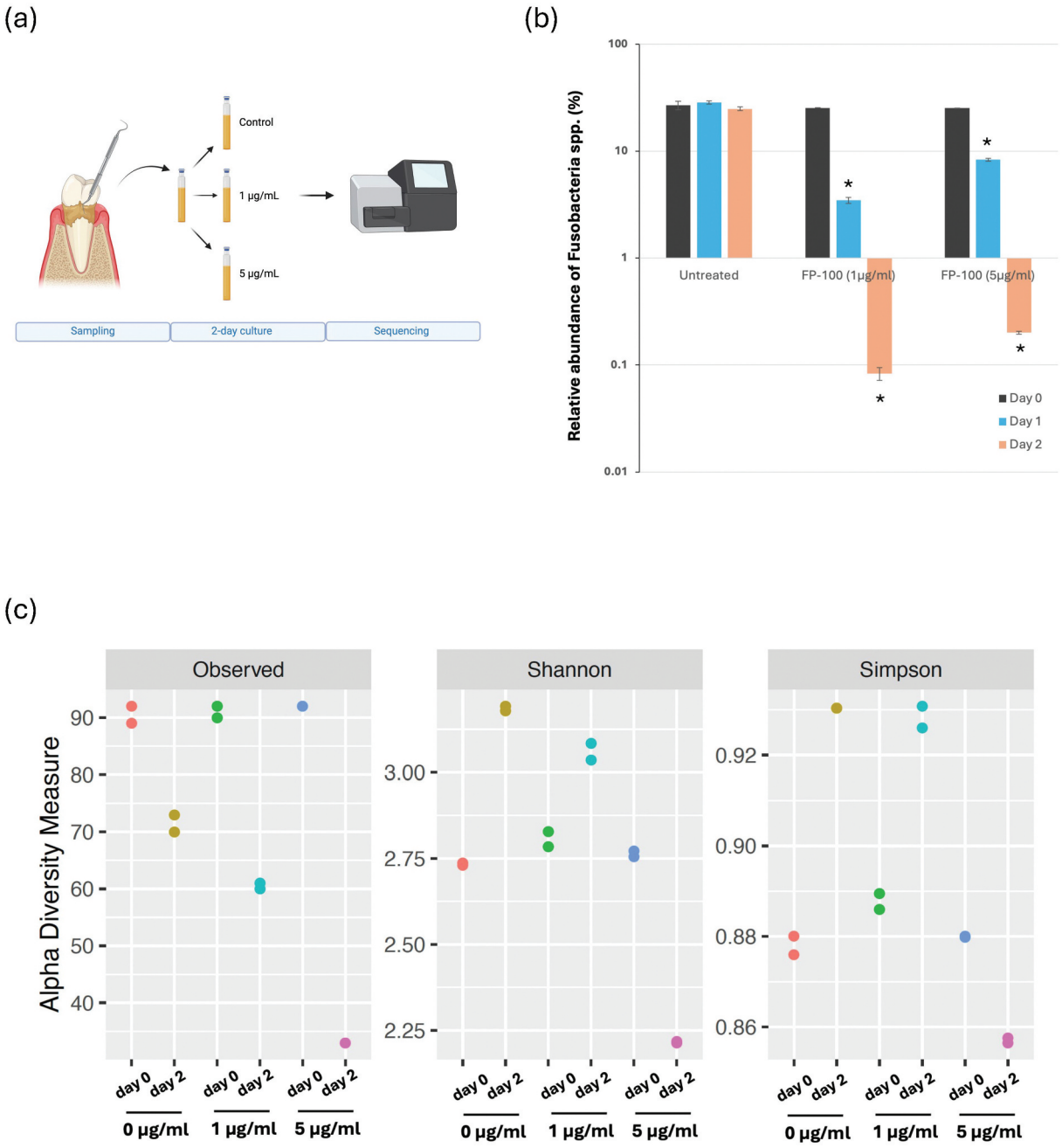

Figure 1. Efficacy of FP-100 on the multispecies microbial community in an *in vitro* model. At a 1 µg/ml concentration, FP-100 reduced the relative abundance of *F. nucleatum* without significantly altering the alpha diversity. Increasing the concentration to 5 µg/ml yielded no additional benefit in bacterial reduction. (a) The experimental setup, created with BioRender.com, (b) changes in the relative abundance of *F. nucleatum* across days, and (c) alpha diversity graphs at different concentrations over the days.

Table 1. Presence of cultivable *F. nucleatum* in ligature following vehicle or FP-100 treatment. Detection of *F. nucleatum* was performed using species-specific primers polymerase chain reaction (PCR) on a mixed sample of all colonies that grew following 48 h of anaerobic incubation.

F. nucleatum	Vehicle ($n = 10$)	FP-100 ($n = 10$)
Detectable (n)	10/10	0/10

including modified transfer-RNA derived small RNA (tsRNA) [38–42]. Although many of these proposed strategies are still in the *in vitro* testing stage, *in vivo* evaluation in animal models shows promising results in reducing alveolar bone loss. For example, gold nanoclusters have been shown to reduce inflammation and alveolar bone loss by eradicating *F. nucleatum* from dental plaque [37]. Additionally, probiotic treatment against *F. nucleatum* with *Akkermansia municiphila* has been observed to moderate the periodontal inflammation in a mouse model [40]. FP-100 is emerging as a viable alternative to these various targeted modalities, owing to its ease of application and effectiveness at very low doses.

We employed a mouse model to study *F. nucleatum*-induced periodontitis, as its application

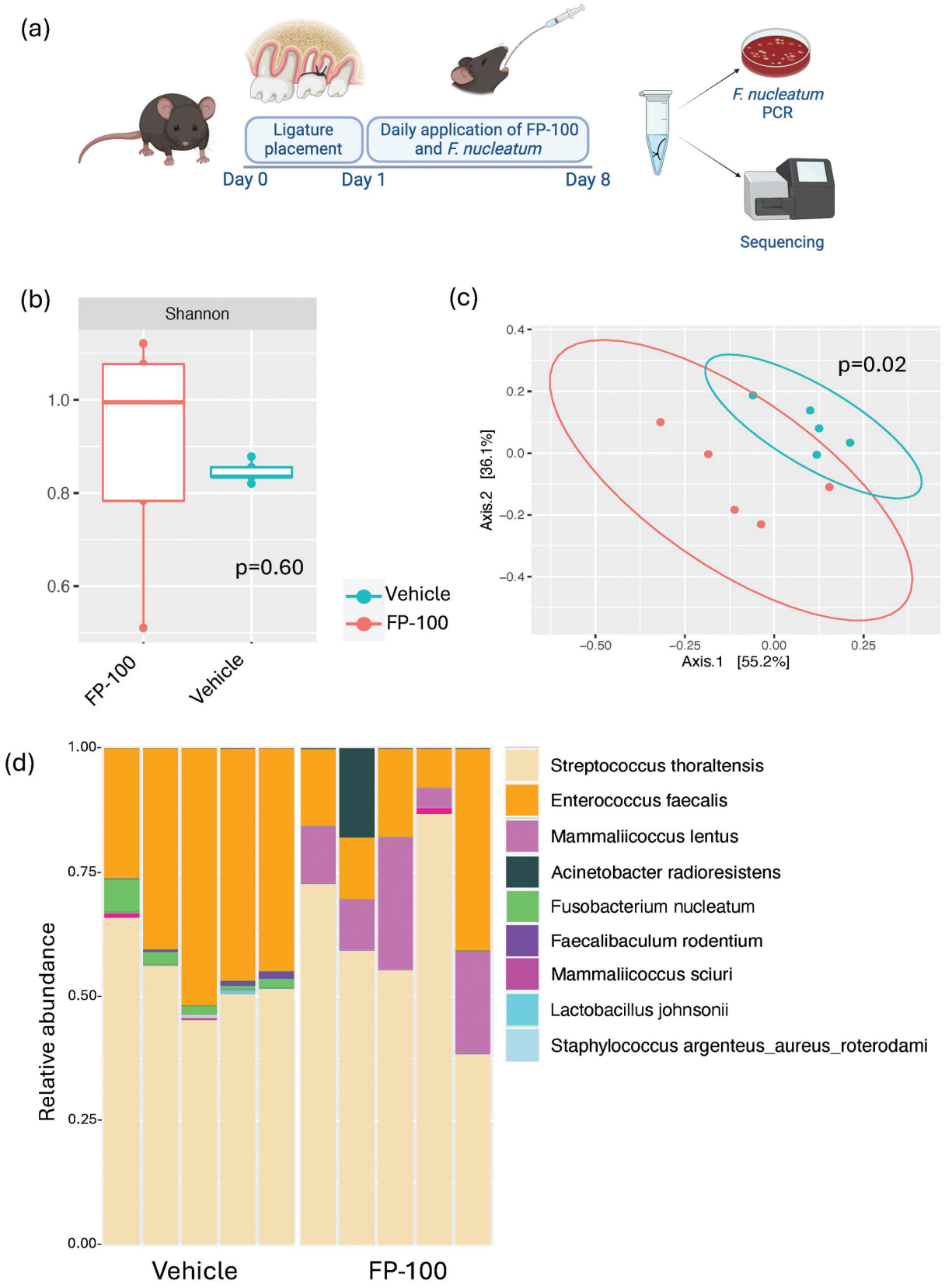

Figure 2. Microbial community analysis using 16S rRNA sequencing. FP-100 treatment reduced the relative abundance of *F. nucleatum* without a significant change in alpha diversity, but a significant community shift was demonstrated by beta diversity. (a) Experimental protocol, created with BioRender.com. (b) Shannon diversity index, given as the representative of all tested alpha diversity indices, shows no difference between treatment and vehicle groups ($n = 5$ per group). (c) Principal coordinates analysis (PCoA) of the bray-Curtis dissimilarity, demonstrating significant differences in community structure between the vehicle and FP-100 groups ($p = 0.02$) ($n = 5$ per group). (d) Comparison of the relative abundances of the most abundant 9 bacterial species (*Streptococcus thoraltensis*, *Enterococcus faecalis*, *Mammaliicoccus lentus*, *Acinetobacter radioresistens*, *Fusobacterium nucleatum*, *Faecalibaculum rodentium*, *Mammaliicoccus sciuri*, *Lactobacillus johnsonii*, and a *Staphylococcus* species ambiguously identified as *S. argenteus*, *S. aureus*, or *S. roterodami*) between the control (vehicle) and FP-100 treated groups.

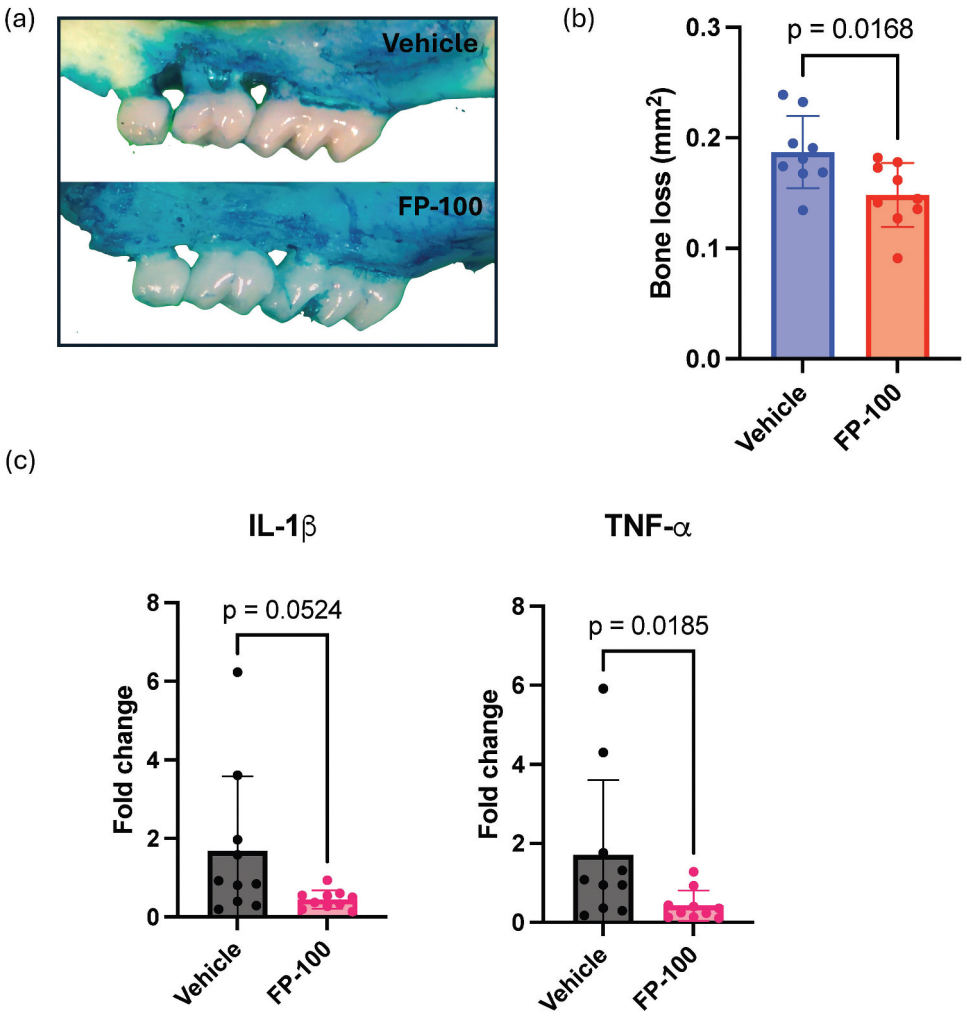

Figure 3. Efficacy of FP-100 in the mouse model of ligature-induced periodontitis. FP-100 effectively eradicated cultivable *F. nucleatum* from the ligature site and significantly mitigated alveolar bone loss in a mouse model of ligature-induced periodontitis. (a) Representative images of maxillary alveolar unit morphology in the control (vehicle) and FP-100-treated groups, and (b) morphometric analysis comparing alveolar bone loss between the two groups ($n = 9$ per group), (c) gingiva RT-PCR results demonstrated higher IL-1β and tnf-α expressions in the vehicle group compared to the FP-100-treated group ($n = 10$ per group). P-values were calculated using the Mann-Whitney test.

has been previously reported to cause bone loss in the alveolar bone or abscesses in murine models [13,43]. Ligature placement around the maxillary molars of mice is known to result in significant bone loss within 7 days [44]. We used silk ligatures for their retentive properties when applying *F. nucleatum*. The antibiotic dose was selected based on the minimum concentration (MIC) necessary to prevent bacterial growth in mice, as identified in the prior study on this compound [19]. This dose of FP-100, administered intraorally, was highly effective against *F. nucleatum* while decreasing the inflammatory response and alveolar bone loss. This compelling observation supports the targeted elimination of a specific pathogenic bacterium, which demonstrates the potency of these novel antibiotic strategies in therapeutic regimens designed to treat infectious diseases.

Increased doses of antibiotics may lead to more intense selection pressure on mutations that confer resistance, potentially resulting in FP-100-resistant *Fusobacterium spp*. Additionally, a higher dose of FP-100 could inhibit the growth of other species that are less sensitive to lower dosages. Both scenarios may have contributed to the increased relative abundance of *Fusobacterium* observed with high-dose FP-100 treatment in our *in vitro* microbial community. As a result, the community changes affected by FP-100 treatment positively impacted disease progression in our mouse model.

In summary, our work shows the efficacy of FP-100 as a strong candidate for its innovative approach, paving the way for a paradigm change.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

NY and OU performed the *in vivo* experiments and their laboratory analyses and wrote the manuscript. LC performed the *in vitro* microbiological analyses, HH collected the clinical samples, and TC performed the bioinformatic analyses. WS, XH, and AK designed the study and revised the manuscript. All authors approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author (AK).

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