Antibiotic protection assay revisited: Metronidazole is unable to completely eliminate *Porphyromonas gingivalis*

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ABSTRACT

Intracellular bacterial survival is widely studied in host-microbiome interactions. The antibiotic protection assay is often used to quantify intracellular bacteria. This method uses gentamicin to kill extracellular bacteria, where the bacteria that remain inside host cells survive the treatment. However, gentamicin is ineffective against anaerobic bacteria, such as *Porphyromonas gingivalis*. To remedy this, metronidazole is often incorporated. However, the effectiveness of this adaptation seems not to be validated properly. The aim of this study was to show the ineffectiveness of metronidazole to eliminate extracellular *P. gingivalis in vitro*. Microscopy showed uptake of *P. gingivalis* by murine J774A.1 macrophages and primary human macrophages. However, quantification of intracellular bacteria was unreliable as the control without macrophages contained significant numbers of viable bacteria. Upon testing metronidazole under assay conditions, *P. gingivalis* survived within the tested timeframes. Next, it was attempted to find a suitable alternative antibiotic compound to use in the antibiotic protection assay. The MIC and MBC were therefore determined for various alternative antibiotics and antimicrobial peptides. None of the included antibiotics effectively killed *P. gingivalis*. Antimicrobial peptides cycloLL-37 and D-LL-31 were effective against *P. gingivalis*, but were toxic to macrophages under the conditions used as determined using a Lactate dehydrogenase-based cytotoxicity assay. To conclude, metronidazole and gentamicin seem unsuitable for the antibiotic protection assay for the strict anaerobic oral bacterium *P. gingivalis*. To be able to determine viable intracellular *P. gingivalis*, alternative bactericidal agents should be found which, under assay conditions, eliminate extracellular *P. gingivalis* without entering or affecting mammalian cells.

1. Introduction

The healthy human body is colonized by microbes that represent our commensal microflora. The number of microbes is estimated to be at least equal to the number of human cells in the body (Sender et al., 2016). Within this microflora, opportunists and pathogens reside without causing an infection. This is due to the effectiveness of our immune system, and the skin and mucosal barriers as the first line of defense. However, occasionally these microbes penetrate these barriers, invading the body, leading to infection and inflammation. When this happens, phagocytic cells can accumulate and inactivate these invading microbes, effectively neutralizing the infection. However, certain microbes can hide within epithelial cells, or survive within and escape from phagocytes preventing their inactivation (Zheng et al., 2021; Werheim et al., 2020; Guidi-Rontani, 2002). Survival within and escape from host cells are therefore important virulence factors of microbes and commonly studied.

Studies on determining bacterial invasion into host epithelial cells, phagocytosis by host immune cells, or survival within host cells often use an antibiotic protection assay by which the number of viable

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bacteria within a host cell is determined (Kaneko et al., 2016). A step-by-step schematic depiction of the antibiotic protection assay is presented in Fig. 1.

Usually, gentamicin is used to kill a variety of bacterial species without entering mammalian cells due to its high molecular weight (Kaneko et al., 2016). After killing the extracellular bacteria, including those that adhere to the cell surface, the host cells are lysed using saponin or demineralized water. Intracellular bacteria are then released, and survival and viability can be quantified using determination of colony forming units (CFU). Surviving bacteria have either actively invaded the host cells (*e.g.* epithelial cells) or were phagocytosed by immune cells (*e.g.* macrophages) and survived within these cells for the duration of the assay.

Gentamicin is an aminoglycoside antibiotic that is effective against a wide range of bacteria (Phillips et al., 1977). It has been applied to study invasion, phagocytosis and intracellular survival *in vitro* of many pathogenic bacteria, most notably the pathogens *Staphylococcus aureus* and *Enterococcus faecalis/faecium* (Rollin et al., 2017; Millan et al., 2013). While membrane impermeable in mammalian cells (Vaudaux and Waldvogel, 1979), gentamicin enters bacterial cells in a oxygen-dependent manner (Bryan et al., 1979). Once inside the cell, gentamicin binds irreversibly to the 16S ribosomal RNA, disrupting protein synthesis which leads to the formation of truncated or non-functional proteins (Weisblum and Davies, 1968; Davis, 1987; Fourmy et al., 1996). Due to its dependency on oxygen to enter bacteria, gentamicin is ineffective against strict anaerobic bacteria such as *Clostridium perfringens*, *Bacteroides fragilis* and *Porphyromonas gingivalis* (Bryan et al., 1979; Schlessinger, 1988; Maezono et al., 2011).

P. gingivalis is an anaerobic, Gram-negative oral bacterium that is mostly associated with periodontitis (Darveau et al., 2012). It has the ability to invade gingival epithelial cells (Lamont et al., 1995; Andrian et al., 2004), to persist within macrophages and even escape them (Werheim et al., 2020; Giacona et al., 2004; Lam et al., 2016). The ability of *P. gingivalis* to invade epithelial cells and persist in phagocytes are essential elements for the mechanism by which *P. gingivalis* passes tissue barriers and travels through the human body (de Jongh et al., 2023). The viability of *P. gingivalis* inside host cells is relevant for studying the association of this bacterium with various diseases in other places in the body, such as cardiovascular diseases, various types of cancer and Alzheimer's Disease (Bui et al., 2019). To study the invasion or uptake of *P. gingivalis*, the antibiotic protection assay should make use of an alternative antibiotic that kills anaerobic bacteria. Gentamicin does not have an inhibitory effect on *P. gingivalis* and is therefore not suitable to use *(Maezono et al., 2011)*. In studies using the antibiotic protection assay to observe internalized *P. gingivalis*, metronidazole is often used in combination with gentamicin, without any evidence that this is an effective strategy. The experimental procedures in these studies lack controls to check for the *in vitro* antimicrobial effectivity of metronidazole and gentamicin on *P. gingivalis* (Lamont et al., 1995; Lam et al., 2016; Farrugia et al., 2021; Katz et al., 2000; Walter et al., 2004; Blancas-Luciano et al., 2024; Soto et al., 2022; Han et al., 2000). Metronidazole is effective against a range of anaerobic bacteria by damaging the bacterial DNA (Pankuch et al., 1993). It enters bacterial cells by diffusion in an inactive form, gets activated by reduction of its nitro group, resulting in generation of reactive oxygen (Dingsdag and Hunter, 2018; Edwards, 1993; Church and Laishley, 1995). For this to happen, *P. gingivalis* needs to be metabolically active. The presence of oxygen decreases the generation of activated metronidazole and increases the regeneration to the inactive form (Dingsdag and Hunter, 2018). In addition, the uptake of metronidazole into the bacterium is inhibited by the presence of oxygen (Church and Laishley, 1995). The antibiotic protection assay is always performed under normal culture conditions for host cells, where oxygen is present (Werheim et al., 2020; Lamont et al., 1995; Giacona et al., 2004). Therefore, it seems unlikely that either gentamicin or metronidazole are able to kill *P. gingivalis* in the current condition of the standard antibiotic protection assay.

An additional issue is that there is no scientific proof that metronidazole cannot enter the host cell. The molecular weight of metronidazole is 171.16 g/mol, which is below the generally accepted limit of 400 g/mol of molecules that cannot pass across plasma membranes (Kaneko et al., 2016). In fact, there is evidence that metronidazole can passively diffuse into human gingival fibroblasts (Yu et al., 2009).

As an alternative to metronidazole and gentamicin, antimicrobial peptides (AMPs) could be a viable alternative to antibiotics to eliminate *P. gingivalis*. These peptides occur naturally in humans and are expressed by epithelial cells of the gut or the gingiva. For example, cathelicidins comprise a family of AMPs that disrupt the cytoplasmic membrane of microorganisms, with members such as LL-31 and LL-37 (Hooper, 2015). By organic synthesis, AMPs can be altered to improve their function. For example, the D-enantiomer of LL-31 (D-LL-31) and the cyclic variant of LL-37 (cycloLL-37) were found to be more effective in eliminating pathogens (Wongkaewkhiaw et al., 2020; White et al., 2022). This is because they are more resistant against degradation by proteolytic enzymes, including *P. gingivalis*-derived proteases (Guo et al., 2010). Other synthetic antimicrobial peptides include DP-7, LF-chimera and Nal-P-113 and have been shown to be effective against multiple bacterial species *in vitro* (Wu et al., 2014; Jiang et al., 2022; Haney et al., 2012; Ruangcharoen et al., 2017; Wang et al., 2018).
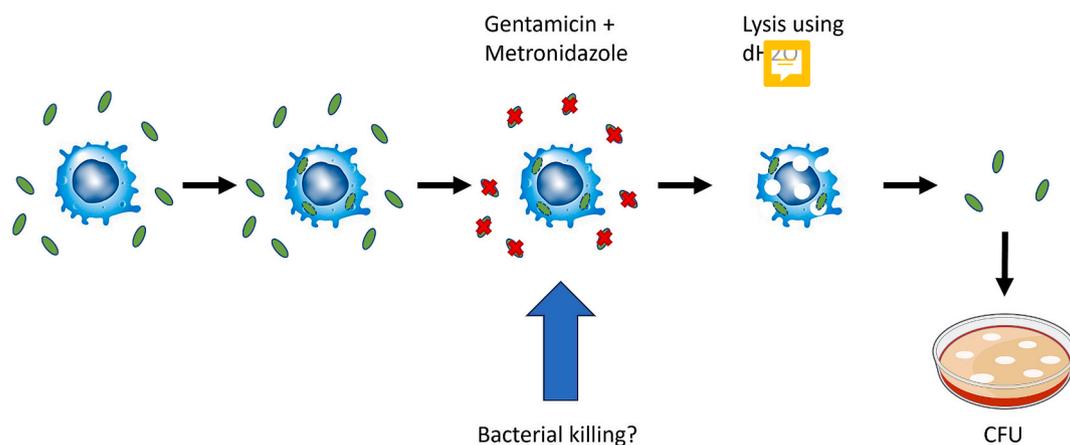

Fig. 1. Schematic depiction of the antibiotic protection assay.
The first step is allowing the host cell (*e.g.* macrophage) to internalize the bacteria. Then, extracellular bacteria are killed using antibiotic treatment. These antibiotics should not be able to enter the host cell. Next, after washing, the host cells are lysed to release the intracellular bacteria, followed by CFU counting to determine the number of surviving (*i.e.* viable) bacteria.

The aim of this study was to evaluate the antimicrobial effects of gentamicin and metronidazole in the *in vitro* antibiotic protection assay on *P. gingivalis*. Besides, this study aimed to develop a suitable version of the antibiotic protection assay using AMPs that can be used for the quantification of viable, host cell internalized *P. gingivalis*. Both murine and human macrophages were used as a model for host cells in this study. Controls were included where no host cells were added, to see whether *P. gingivalis* could survive the entire procedure of the antibiotic protection assay.

2. Materials and methods

2.1. Bacterial culture conditions

P. gingivalis strains W83 and ATCC 33277 were grown as described previously (de Jongh et al., 2024). Briefly, isolates from −80 °C glycerol stocks were cultured on Anaerobic Blood Agar (ABA), which consisted of Tryptic Soy Agar (TSA, Becton Dickinson, Franklin Lakes, USA) supplemented with 2 mg/mL glucose (Merck, Darmstadt, Germany), 5 µg/mL hemin (Sigma-Aldrich, St. Louis, USA) and 1 µg/mL menadione (Vitamin K, Sigma-Aldrich) and 5 % defibrinated sheep blood (Biotrading, Mijdrecht, the Netherlands). Pre-cultures were prepared overnight in Brain Heart Infusion (BHI, Becton Dickinson) broth supplemented with 5 µg/mL hemin and 1 µg/mL menadione (BHI + H/M). *P. gingivalis* was cultured at 37 °C under anaerobic conditions using airtight jars filled with an anaerobic gas mixture: 10 % H_2, 10 % CO_2, 80 % N_2. Culturing *P. gingivalis* on ABA plates was done for 5–7 days and liquid pre-cultures were made one day before an experiment.

2.2. Host cell culture conditions

J774A.1 (ATCC TIB-67) murine macrophages were cultured at 37 °C/5 % CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher, Waltham, Massachusetts, USA) supplemented with 10 % Fetal Bovine Serum (FBS, Sigma-Aldrich) and Antibiotic Antimycotic solution (PSF; 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL Amphotericin B, Sigma-Aldrich) as described previously (Van Dyck et al., 2020). The cells were harvested using a cell scraper and centrifuged at 100 x*g* for 7 min at room temperature before resuspending in DMEM with 10 % FBS, without PSF.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats containing blood from healthy donors (Sanquin, Amsterdam; permission number NVT230.01) as described previously (Ceylan et al., 2024). Magnetic-activated cell sorting (MACS, Miltenyi Biotech, Bergisch Gladbach, Germany) was used to isolate CD14+ monocytes, also as described previously (Davison et al., 2014). The CD14+ cells were resuspended in DMEM supplemented with 10 % FBS and PSF, and 50 ng/mL macrophage colony-stimulating factor (MCSF, R&D systems, Minneapolis, USA) was added for 5–7 days at 37 °C/5 % CO_2 to differentiate them into macrophages. Macrophages were harvested using ice-cold 5 mM Ethylenediaminetetraacetic acid (EDTA, Merck) in PBS and a cell scraper, before centrifuging the cell suspension at 300 x *g* for 10 min. The macrophages were resuspended to the desired cell density using DMEM with 10 % FBS, without PSF.

2.3. Quantifying phagocytosis using the antibiotic protection assay

The antibiotic protection assay was performed as described previously (Werheim et al., 2020; Lamont et al., 1995) with minor modifications. In contrast to most antibiotic protection assays, the bacterium was allowed to adhere first and then the macrophages were added. This was done because it was easier to observe uptake by microscopy as all the bacteria are in the same plane, and to investigate the effectiveness of the antibiotics against the bacteria that are not washed away.

The antibiotic protection assay was performed as follows: A suspension of *P. gingivalis* with a final density of $OD_{600} = 0.05$ (equal to

approx. 10^7 CFU/mL) was prepared by diluting an overnight liquid pre-culture in BHI + H/M. 1 mL of *P. gingivalis* suspension was added to 24 well cell culture plates and the bacterium was allowed to adhere for 2 h at 37 °C under normal atmospheric oxygen levels (normoxic conditions) on a rocking platform before washing with PBS. Then, macrophages were harvested as described above and added at a density of $5*10^4$ cells/mL to the wells which contained *P. gingivalis*. The macrophages were incubated for 1 h at 37 °C under normoxic conditions with added 5 % CO_2 to allow for phagocytosis. Then, the killing medium (DMEM with 10 % FBS and 200 µg/mL of metronidazole) was added and incubated for various timepoints under the same conditions as the previous step. After incubation cells were lysed using demineralized water and the lysate was removed from the wells by scraping with a pipette tip. Samples were 10-fold serially diluted and spread over ABA plates. These plates were incubated under anaerobic conditions at 37 °C for 5–7 days and the number of colony forming units (CFU) was determined. Controls were performed on only *P. gingivalis* without addition of macrophages, to confirm that the extracellular bacteria were eliminated.

In later experiments, the effectiveness of the combination of metronidazole (200 µg/mL) and gentamicin (300 µg/mL) was tested in the same setup, without macrophages. After that, the antibiotics in Table 1 were tested in the described concentrations, but this time the samples were washed using centrifugation (1400 x*g* for 5 min) after antibiotic treatment instead of washing with PBS to limit the loss of bacteria during washing. This antibiotic treatment was done for 3 h, instead of 1 h to investigate whether a prolonged treatment would be more effective.

To image the uptake of *P. gingivalis* by macrophages, time-lapse microscopy was performed after addition of the macrophages. To visualize *P. gingivalis*, the bacterium was stained using carboxyfluorescein succinimidyl ester (CFSE, Sigma-Aldrich) in PBS for 30 min at 37 °C, as described previously (de Jongh et al., 2024). This staining was performed before the macrophages were added. Afterwards, *P. gingivalis* was washed twice with PBS by centrifuging for 1 min at 21300 x *g*. Bacteria were allowed to adhere and then macrophages were added as described above and the cells were imaged using the Axio Observer Zeiss Z1 microscope (Zeiss, Oberkochen, Germany) with a 20× objective. IL-4 (10 ng/mL, R&D systems) was added to the primary macrophages 48 h before adding them to the wells containing *P. gingivalis* for further differentiation. Both brightfield and green fluorescent (Excitation filter: 475/40 Emission filter: 530/50) channels were imaged every 10 min for 18 h. Composite images of brightfield and fluorescence pictures, including scalebars and timestamps, were made using ImageJ (version 1.54 g) (Schneider et al., 2012).

2.4. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined as described previously (Wiegand et al., 2008). In short, a two-fold serial dilutions of antibiotic or the alternative antimicrobial peptides (AMPs) was added to a suspension of *P. gingivalis* with a final density of $OD_{600} = 0.001$, which was prepared by diluting an overnight liquid culture in BHI + H/M. Bacteria were incubated with antibiotics/AMPs under anaerobic conditions at 37 °C for 48 h (strain ATCC 33277) or 72 h (strain W83). The lowest

Table 1
Antibiotics and concentrations used in this study.

Antibiotic	Concentration used	Manufacturer
Metronidazole	200 µg/mL	Sigma-Aldrich
Gentamicin	300 µg/mL	Sigma-Aldrich
Faropenem	300 µg/mL	Cayman Chemicals
PSF: Penicillin, streptomycin & Amphotericin B	100 U/mL, 100 µg/mL & 250 ng/mL, respectively	Sigma-Aldrich

concentration of antibiotic or AMP that showed no visible growth of bacteria was determined to be the MIC. The contents of the wells were resuspended by pipetting and the MBC was determined by taking 50 μL of each well without visible growth and cultured on ABA as described above. The lowest concentration that showed no colonies on the ABA plates was determined to be the MBC. The experiment was performed at least two times to determine ranges for the MIC and MBC for both strains. Controls without bacteria were taken to check for sterility and a range of dimethylsulfoxide (DMSO, Sigma-Aldrich) concentrations was used as a vehicle control for metronidazole.

2.5. Antimicrobial peptides

The AMPs used in this study are listed in Table 2. All peptides expect cycloLL-37 were synthesized and purified as previously described (Soldati et al., 2023). In short, solid-phase peptide synthesis was performed using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry with a Siro II synthesizer (Biotage, Uppsala, Sweden). Cyclo-LL-37 was prepared using click chemistry as described (Arias et al., 2014). For this, the linear peptide (sequence: Fmoc-Bishomoproparglyglycine-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES -Fmoc-Azidolysine) was produced. The cyclization was performed on resin; to 4 eq. peptide in a 20 mL syringe, 8 eq. 2,6-lutidine, 8 eq. 2,2-bipyridine, 4 eq. CuBr and 8 eq. of sodium ascorbate (all peptide grade, Actu-All Chemicals, Oss, The Netherlands) were added and flushed for 1 min with N_2. After incubation for 24 h at room temperature, the mixture was flushed sequentially by DMF, H_2O, MetOH, EDTA (100 mM), H_2O and DMF and subsequently dried by flushing three times with isopropanol and DCM. Next, the cyclized peptide was detached from the resin. The peptides were then purified *via* HPLC using a Dionex Ultimate 3000 system (Thermo Scientific, Breda, The Netherlands). Their authenticity was confirmed through mass spectrometry with a Microflex LRF MALDI-TOF (Bruker Daltonik GmbH, Bremen, Germany). Finally, the peptides were dissolved in Milli-Q water at a concentration of 10 mM and stored at −20 °C until use.

2.6. Cytotoxicity assay

Lactate dehydrogenase (LDH) release in the supernatant of J77A.1 macrophages as an indicator of cytotoxicity after exposure to AMPs for 1 h was measured using the Cytotoxicity LDH kit plus (Roche, Bazel, Switzerland) according to the manufacturer's instructions. The background was corrected with a medium-only control (DMEM +5 % FBS) and a positive control was included by lysing the cells using 2 % Triton X-100 (Merck). The absorbance was measured at 490 nm using a Spectramax M2 spectrophotometer (Molecular Devices, San Jose, USA). Cytotoxicity was expressed as the percentage of the averaged Triton-X-100 positive control. In the samples where the OD value was higher than that of the positive control, the level of cytotoxicity was set to 100 %.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 10.1.0 for Windows, GraphPad Software, San Diego, California USA,

www.graphpad.com). Unpaired *t*-tests were performed to determine statistical difference between CFU counts of samples that were incubated with or without macrophages. For the comparison of multiple antibiotics, an ANOVA with post-hoc Tukey test was performed with a significance level (α) of 0.05. For each experiment, biological duplicates were performed, and the number of technical replicates is depicted in each figure by the number of symbols per column.

3. Results

3.1. Phagocytosis of P. gingivalis by macrophages

Macrophages were allowed to phagocytose adherent *P. gingivalis* overnight. Live-cell imaging showed that fluorescently labeled *P. gingivalis* were taken up by J774A.1 murine macrophages (Fig. 2A) and primary human macrophages (Fig. 2B). Already after 1 h, some phagocytosis was observed. In order to quantify the number of *P. gingivalis* that are taken up by these macrophages, the antibiotic protection assay was performed. The results of this assay show that up to 100 CFU/mL of *P. gingivalis* were present in the samples after addition of both human primary macrophages (Fig. 2C) and murine J774A.1 macrophages (Fig. 2D), suggesting that this number of *P. gingivalis* were taken up by the macrophages. However, the controls without any macrophages still contained live *P. gingivalis* (Fig. 2C, D). This means that metronidazole did not effectively kill the extracellular *P. gingivalis* in the setup for the antibiotic protection assay.
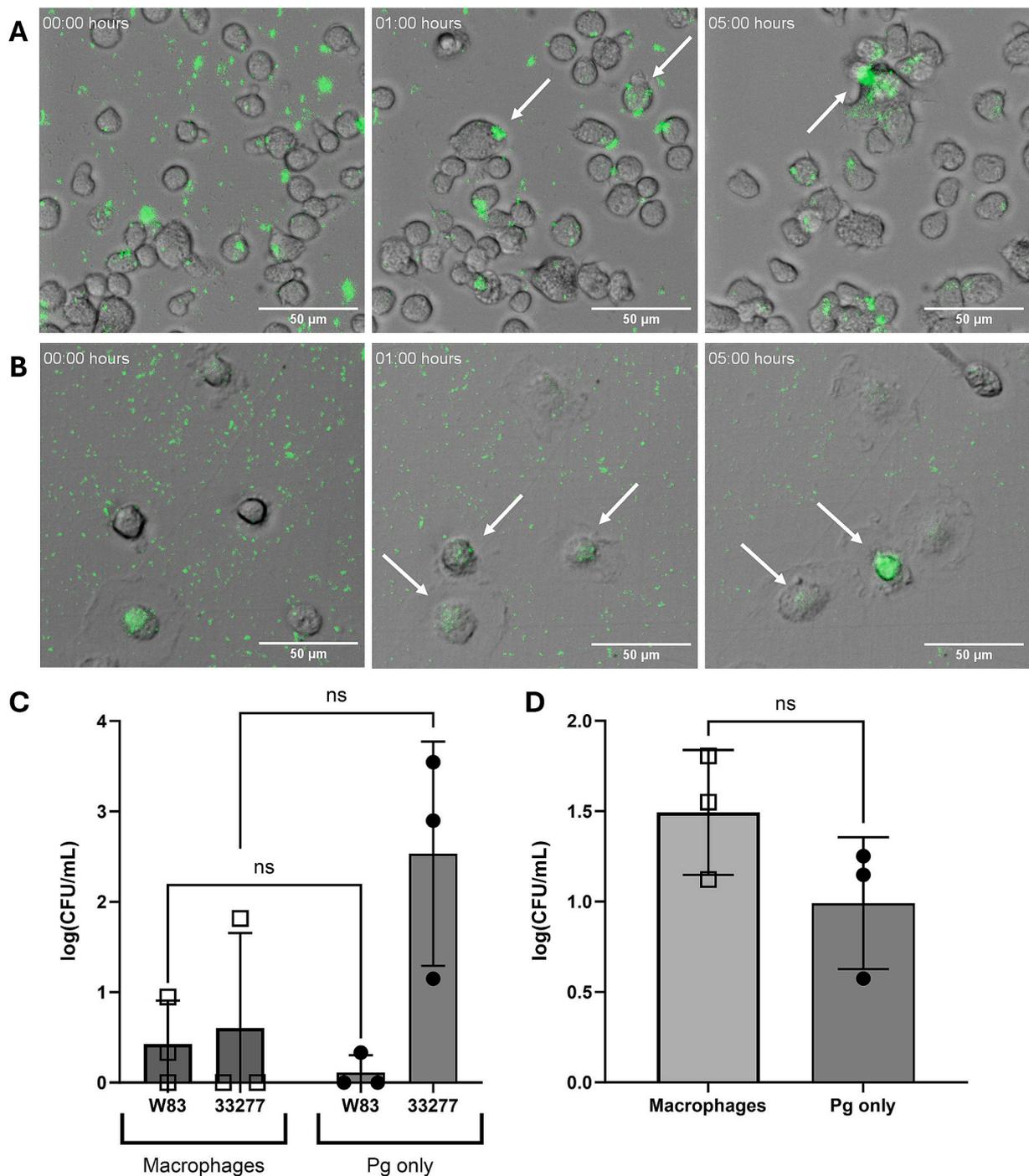
3.2. MIC and MBC determination of various antibiotics

The MIC of various antibiotics on two different strains of *P. gingivalis* was determined as seen in Table 3. From there, the MBC was determined. According to these results, a concentration of 50 μg/mL of metronidazole would be sufficient to kill *P. gingivalis*. The concentration of DMSO that is used as a solvent for metronidazole did not affect the growth of *P. gingivalis* (data not shown). Faropenem has an MBC range of 0.5–1 μg/mL, as derived from literature (Table 3). No MBC could be determined for gentamicin within the tested range, and no MBC could be found for gentamicin and penicillin in literature.

The combination of gentamicin and metronidazole was also tested under assay conditions. The assay was performed as described, but no macrophages were added. As seen in Fig. 3A, this combination did not affect the viability of *P. gingivalis* after 1 h of incubation and washing afterwards. Both strains of *P. gingivalis* were unaffected. Next, the bactericidal activity of the antibiotics was tested after washing using centrifugation. From the results in Fig. 3B, it can be seen that the antibiotics did not inactivate *P. gingivalis*. Even after 3 h of incubation, with high concentrations of antibiotics (200 μg/mL Metronidazole, 300 μg/mL faropenem or 300 μg/mL gentamicin), none of the tested antibiotics resulted in complete killing of *P. gingivalis*.

3.3. MIC and MBC determination of various AMPs

To find a suitable alternative for the antibiotics, several AMPs were tested for their efficacy in inhibiting *P. gingivalis* growth and killing. The results are shown in Table 4. Cyclo-LL-37 and D-LL-31 were able to eliminate *P. gingivalis* ATCC 33227. According to these results, a concentration of 55.64 μM cycloLL-37 and a 50 μM concentration of D-LL-31 should be sufficient to eliminate *P. gingivalis* ATCC 33277. *P. gingivalis* strain W83 was more tolerant to D-LL-31, as no MBC was found. For the other AMPs, no MBC could be found for *P. gingivalis*. Strain ATCC 33277 seemed more resistant to the AMPs as for three of the seven tested AMPs no MIC could be found, whereas for strain W83 a MIC could be found but no MBC.

Table 2
Antimicrobial peptides used in this study.

Antimicrobial peptide	Amino acid sequence
DP7	VQWRIRVAVIRK
LL31 / D-LL-31	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL
Cyclo-LL-37	*LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES*
Nal-P-113	Ac-AKR-Nal-Nal-GYKRKF-Nal-NH$_2$
LF-chimera	DLIWKLLSKAQEKFGKNKSR
	|
	FKCRRWQWRMKKLG —K

Fig. 2. Phagocytosis of *P. gingivalis* by J774A.1 murine macrophages and primary human macrophages.
CFSE-labeled *P. gingivalis* ATCC 33277 (green) were phagocytosed by J774A.1 macrophages (A) and *P. gingivalis* W83 was phagocytosed by primary human macrophages (B). After 5 h green fluorescent *P. gingivalis* were taken up as they were now localized in the macrophages instead of on the surface, an area without fluorescent *P. gingivalis* was observed around the indicated primary macrophages. The white arrows indicate the macrophages that were actively taking up *P. gingivalis*. The graphs below show the quantification of uptake by J774A.1 macrophages (C) and primary human macrophages (D). Number of viable *P. gingivalis* after treatment (Log10 CFU/mL) with 200 μg/mL metronidazole and lysing the cells. Total viable bacteria were not significantly different with or without macrophages added, suggesting that the treatment with metronidazole was not killing the bacteria. For the primary macrophages, only strain W83 was used. Each symbol represents the average of the intra-experimental duplicates of each independent experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.4. Cytotoxicity of AMPs to macrophages

Next, cytotoxicity of these AMPs to host cells was determined (Fig. 4). The aim was to find an AMP that can eliminate *P. gingivalis* without affecting the host cell, and so the AMPs were tested in the same concentration range as for the MIC/MBC determination. The J77A.1 murine macrophage cell line was chosen as a model host cell to determine cytotoxicity. The MBC of cycloLL-37, determined to be 55.64 μM was cytotoxic to macrophages, with a LDH release of around 60 %. D-LL-31 (50 μM) is also cytotoxic, with LDH release of around 50 %. LL-31, the

Table 3
MIC and MBC of various antibiotics on *P. gingivalis* under anaerobic conditions.

| Antibiotic | W83 | | ATCC 3277 | | Clinical isolates* | | |
	MIC range	MBC range	MIC range	MBC range	MIC range	MBC range	Reference
Metronidazole	≤3.13	6.25–50	≤3.13	≤3.13	0.016–2	0.125–2	(Larsen, 2002; Milazzo et al., 2003; Kulik et al., 2008; Conrads et al., 2021; Wright et al., 1997; Andres et al., 1998)
Gentamicin	>300	–	>300	–	–	–	
Faropenem	–	–	–	–	≤0.03–0.5	0.5–1	(Milazzo et al., 2003)
Penicillin	–	–	–	–	≤0.03–1	–	(Andres et al., 1998)

MIC and MBC are expressed in µg/mL. –: Not tested nor reported in literature. The MIC and MBC ranges of W83 and ATCC 33277 are based on the results of at least two independent experiments. *: The ranges of the clinical isolates were derived from literature and not empirically established in the present study.
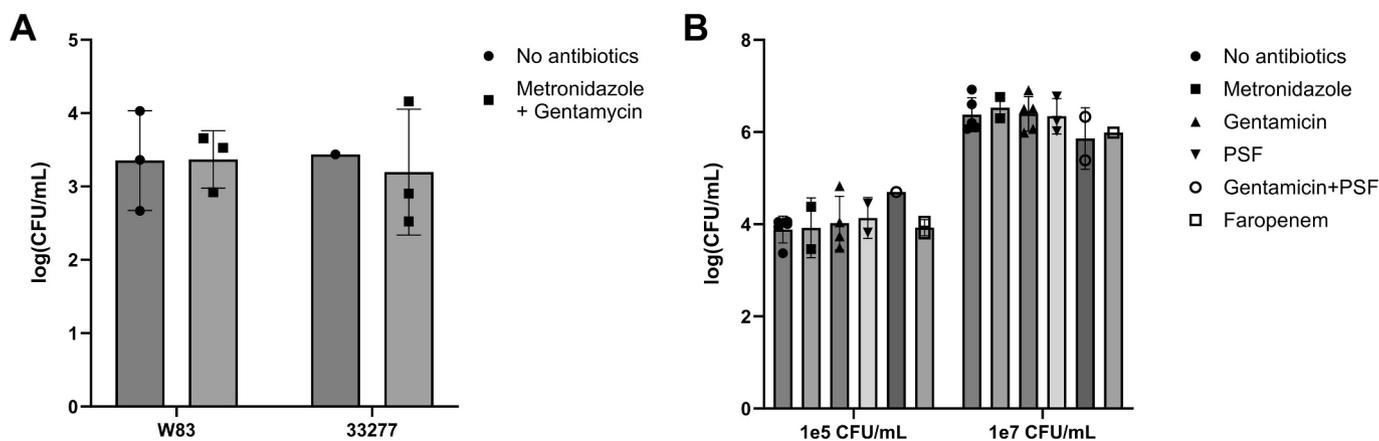
Fig. 3. Determining the killing efficiency of various antibiotics after washing. (A) The number of viable *P. gingivalis* after treatment with Metronidazole (200 µg/mL) and Gentamicin (300 µg/mL) for 1 h and washing with PBS, without centrifugation, following the entire protocol for the antibiotic protection assay. The inoculum density was 10^7 CFU/mL. (B) The number of viable *P. gingivalis* (strain ATCC 33277) after 3 h of treatment with various alternative antibiotics and washing using centrifugation. Two inoculum densities of *P. gingivalis* ATCC 33277 were tested, 10^5 CFU/mL and 10^7 CFU/mL. In both graphs, there were no differences between the control and the samples that were treated with antibiotics. Each symbol represents the average of the intra-experimental duplicates of each independent experiment. No statistical differences were found between the tested antibiotics and the control without antibiotics.

Table 4
MIC and MBC of several AMPs for *P. gingivalis* after 48–72 h under anaerobic conditions.

| Antimicrobial peptides | *P. gingivalis* W83 | | *P. gingivalis* ATCC 33277 | |
	MIC range	MBC range	MIC range	MBC range
DP-7 (µM)	>50	–	>50	–
LL-31 (µM)	12.5	–	>50	–
D-LL-31 (µM)	3.13	–	3.13	12.5–50
Cyclo-LL-37 (µM)	1.74–55.64	55.64	6.96–13.91	55.64
Nal-P-113 (µM)	3.13	–	12.5–50	–
LF-chimera (µM)	3.13	–	>50	–

MIC and MBC are expressed in µM. –: Not tested or no bactericidal effect could be observed within the tested range. The MIC and MBC ranges are based on the results of two independent experiments.

natural form of D-LL-31, was the only AMP tested that was not found to be cytotoxic to J774A.1 murine macrophages up to a concentration of 500 µM.

4. Discussion

For any antibiotic protection assay to work there are two requirements: First, the antibiotics should completely eliminate *P. gingivalis* within the incubation time under assay conditions. Second, the antibiotics should not be actively transported or passively diffused across the host cell membrane, or affect the viability of the internal bacteria. In studies that use the antibiotic protection assays for *P. gingivalis*, it seems that it is broadly assumed that metronidazole is able to eliminate *P. gingivalis* in 1 h (Lamont et al., 1995; Giacona et al., 2004; Lam et al., 2016; Walter et al., 2004). However, no data is reported to prove these statements (Werheim et al., 2020; Lamont et al., 1995). It cannot be said that metronidazole effectively eliminates extracellular *P. gingivalis* as none of the studies have taken proper controls to show that it was successful. Therefore, first aim of this study was to investigate the efficacy of metronidazole and gentamicin to eliminate *P. gingivalis* under the conditions of the antibiotic protection assay.

The present study shows that metronidazole and gentamicin do not eliminate *P. gingivalis* after visible internalization by macrophages, meaning the first requirement of the antibiotic protection assay is not met. None of the other tested antibiotics, gentamicin, faropenem or a combination of penicillin and streptomycin, were able to effectively eliminate *P. gingivalis*. Next, in-house synthesized AMPs were tested. While cycloLL-37 and D-LL-31 were found to be effective in reducing the viability of *P. gingivalis*, the concentration that was required to kill the bacterium also appeared to be cytotoxic to macrophages.

The incorporation of metronidazole is an inherent flaw in the redesign of the method, as the assay is performed in the presence of oxygen which renders metronidazole ineffective by decreasing its activation and inhibiting entry into the bacterium (Dingsdag and Hunter, 2018). In addition, it can be expected that metronidazole diffuses across the plasma membrane of mammalian cells, meaning it will not exclusively affect extracellular bacteria (Yu et al., 2009).

Some studies performed controls by taking a sample of the supernatant during the antibiotics treatment to confirm that the bacteria are no longer viable (Werheim et al., 2020; Eick and Pfister, 2004).
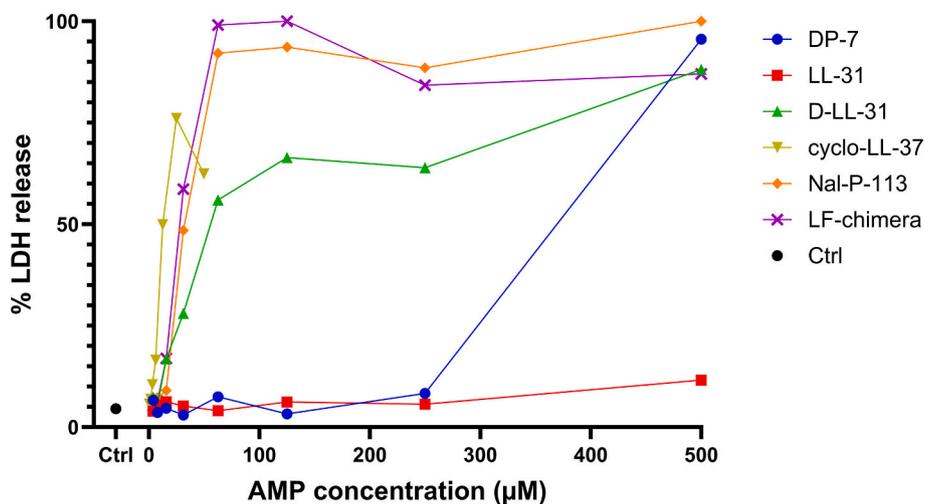
Fig. 4. Cytotoxicity of AMPs to J774A.1 murine macrophages.
J774A.1 murine macrophages were exposed to various concentrations of AMPs for 1 h and their cytotoxicity was determined *via* LDH release. The values were relative to a positive control using Triton X-100.

However, when taking controls like this, the antibiotics are present in extremely high concentrations in the medium, which may be transferred to the agar during plating. The residual presence of antibiotics is likely to inhibit the bacterial growth on the agar (*i.e.* formation of CFU), which means it cannot be concluded that the bacteria were completely eliminated during the initial treatment. This drug carryover effect by metronidazole has been addressed before in a study using *Trichomonas vaginalis*, which showed that if the samples were washed after antibiotic treatment, the concentration of metronidazole that was needed to rapidly kill *T. vaginalis* was more than 5 times higher compared to a previous study (Nix et al., 1995; Krieger et al., 1985). It should be noted that the determination of the MBC is performed in a similar way (Larsen, 2002; Lorenzi et al., 2024; European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical M, Infectious D, 2000). In this case, this might not be as much of an issue as lower concentrations are used which might diffuse out in the agar. In the antibiotic protection assay however, high concentrations of antibiotics are used, making this effect more prevalent. None of the studies that used the antibiotic protection assay have used controls without host cells to validate the bactericidal effect during the entire procedure of the assay (Werheim et al., 2020; Lamont et al., 1995; Lam et al., 2016; Farrugia et al., 2021; Katz et al., 2000; Walter et al., 2004; Blancas-Luciano et al., 2024; Soto et al., 2022; Han et al., 2000). Notably, the current study shows that the bacteria survived the antibiotic treatment under these conditions.

Many studies, including the current study, have shown uptake or invasion of *P. gingivalis* using qualitative microscopic methods, so there is no doubt about whether these phenomena are occurring (Lamont et al., 1995; Lam et al., 2016; Farrugia et al., 2021; Walter et al., 2004). In addition, it should be noted that washing before antibiotic treatment already removes the majority of bacteria, so the numbers that are found in these studies could be in the right order of magnitude. However, the bacteria that adhere to the surface of the host cell or the cell culture plate survive the antibiotic treatment, which results in an overestimation of the survival of *P. gingivalis* inside host cells.

Regarding the AMPs, D-LL-31 was previously tested to have a low cytotoxicity against A549 human lung epithelial cells up to concentrations of 25 μM (Wongkaewkhiaw et al., 2020). While macrophages and lung epithelial cells might not be equally tolerant to the AMPs, it is in line with the results of the current study where the cytotoxicity starts to increase from approximately 30 μM. However, the concentration required to kill *P. gingivalis* is much higher, at around 50 μM. In other research, cycloLL-37 showed a cytotoxic effect on 5637 human

uroepithelial cells at a concentration of 50 μM. In the current study, a cytotoxic effect is already seen at a concentration from around 30 μM in J774A.1 murine macrophages. In any case, the MBC of 55.64 μM for *P. gingivalis* that was found in this study is cytotoxic to host cells. The killing efficiency of D-LL-31 or cycloLL-37 on *P. gingivalis* has not been investigated before.

Combining antibiotics with AMPs might provide a solution (Wongkaewkhiaw et al., 2020; Taheri-Araghi, 2024; Wu et al., 2017). As AMPs disrupt the cell membrane of bacteria, it could allow for antibiotics to enter the bacteria that would normally not be able to enter, such as gentamicin. This could result in requiring a lower concentration of AMPs, so that it would be less cytotoxic to the host cells. It is also important to consider the strain-specific differences of *P. gingivalis*, as these variations can influence the effectiveness of the treatment, as demonstrated in the current study.

In conclusion, the antibiotic protection assay, as currently being used in many studies, is unsuitable to determine phagocytosis or invasion of *P. gingivalis* into host cells, nor its survival within cells. This is an issue that needs to be addressed, as recent studies are still using this method (Werheim et al., 2020; Blancas-Luciano et al., 2024; Soto et al., 2022). Research using the antibiotic protection assay for anaerobes should always incorporate proper controls to show that extracellular microorganisms are indeed eliminated. Findings could be substantiated by using other methods such as fluorescence microscopy or flow cytometry. Studies that base their conclusions on the number of viable internalized *P. gingivalis* are not to be trusted and these experiments should be repeated using a suitable, validated method. Future research on the use of antimicrobial peptides could offer a solution, perhaps in combination with bactericidal antibiotics targeting strict anaerobic Gram-negative bacteria. It is essential that a suitable alternative is found, as the antibiotic protection assay can be used to investigate how pathogenic bacteria such as *P. gingivalis* can persist in host cells, which is important for their invasion into the host, their evasion of our immune system and their influence on various diseases.

CRediT authorship contribution statement

Caroline A. de Jongh: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Volle:** Methodology, Investigation, Formal analysis, Data curation. **Chen Sun:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dongmei Deng:** Supervision, Conceptualization. **Michel A. Hoogenkamp:** Writing – review &

editing, Methodology, Conceptualization. **Kamran Nazmi:** Resources, Methodology. **Floris J. Bikker:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Bastiaan P. Krom:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2025.107214.

Data availability

Data will be made available on request.

References

Andres, M.T., Chung, W.O., Roberts, M.C., Fierro, J.F., 1998. Antimicrobial susceptibilities of Porphyromonas gingivalis, Prevotella intermedia, and Prevotella nigrescens spp. isolated in Spain. Antimicrob. Agents Chemother. 42 (11), 3022–3023.

Andrian, E., Grenier, D., Rouabhia, M., 2004. In vitro models of tissue penetration and destruction by Porphyromonas gingivalis. Infect. Immun. 72 (8), 4689–4698.

Arias, M., McDonald, L.J., Haney, E.F., Nazmi, K., Bolscher, J.G., Vogel, H.J., 2014. Bovine and human lactoferricin peptides: chimeras and new cyclic analogs. Biometals 27 (5), 935–948.

Blancas-Luciano, B.E., Becker-Fauser, I., Zamora-Chimal, J., Jimenez-Garcia, L., Lara-Martinez, R., Perez-Torres, A., et al., 2024. Cystatin C: immunoregulation role in macrophages infected with Porphyromonas gingivalis. PeerJ 12, e17252.

Bryan, L.E., Kowand, S.K., Van Den Elzen, H.M., 1979. Mechanism of aminoglycoside antibiotic resistance in anaerobic bacteria: Clostridium perfringens and Bacteroides fragilis. Antimicrob. Agents Chemother. 15 (1), 7–13.

Bui, F.Q., Almeida-da-Silva, C.L.C., Huynh, B., Trinh, A., Liu, J., Woodward, J., et al., 2019. Association between periodontal pathogens and systemic disease. Biom. J. 42 (1), 27–35.

Ceylan, M., Schoenmaker, T., Hogervorst, J.M.A., Jansen, I.D.C., Schimmel, I.M., Prins, C.M., et al., 2024. Osteogenic differentiation of human gingival fibroblasts inhibits osteoclast formation. Cells 13 (13).

Church, D.L., Laishley, E.J., 1995. Reduction of metronidazole by hydrogenase from clostridia. Anaerobe 1 (2), 81–92.

Conrads, G., Klomp, T., Deng, D., Wenzler, J.S., Braun, A., Abdelbary, M.M.H., 2021. The antimicrobial susceptibility of Porphyromonas gingivalis: genetic repertoire, global phenotype, and review of the literature. Antibiotics (Basel) 10 (12).

Darveau, R.P., Hajishengallis, G., Curtis, M.A., 2012. Porphyromonas gingivalis as a potential community activist for disease. J. Dent. Res. 91 (9), 816–820.

Davis, B.D., 1987. Mechanism of bactericidal action of aminoglycosides. Microbiol. Rev. 51 (3), 341–350.

Davison, N.L., ten Harkel, B., Schoenmaker, T., Luo, X., Yuan, H., Everts, V., et al., 2014. Osteoclast resorption of beta-tricalcium phosphate controlled by surface architecture. Biomaterials 35 (26), 7441–7451.

de Jongh, C.A., de Vries, T.J., Bikker, F.J., Gibbs, S., Krom, B.P., 2023. Mechanisms of Porphyromonas gingivalis to translocate over the oral mucosa and other tissue barriers. J. Oral Microbiol. 15 (1), 2205291.

de Jongh, C.A., Bikker, F.J., de Vries, T.J., Werner, A., Gibbs, S., Krom, B.P., 2024. Porphyromonas gingivalis interaction with Candida albicans allows for aerobic escape, virulence and adherence. Biofilm 7, 100172.

Dingsdag, S.A., Hunter, N., 2018. Metronidazole: an update on metabolism, structure-cytotoxicity and resistance mechanisms. J. Antimicrob. Chemother. 73 (2), 265–279.

Edwards, D.I., 1993. Nitroimidazole drugs–action and resistance mechanisms. I. Mechanisms of action. J. Antimicrob. Chemother. 31 (1), 9–20.

Eick, S., Pfister, W., 2004. Efficacy of antibiotics against periodontopathogenic bacteria within epithelial cells: an in vitro study. J. Periodontol. 75 (10), 1327–1334.

European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical M, Infectious D, 2000. EUCAST Definitive Document E.Def 1.2, May 2000: terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. Clin. Microbiol. Infect. 6 (9), 503–508.

Farrugia, C., Stafford, G.P., Potempa, J., Wilkinson, R.N., Chen, Y., Murdoch, C., et al., 2021. Mechanisms of vascular damage by systemic dissemination of the oral pathogen Porphyromonas gingivalis. FEBS J. 288 (5), 1479–1495.

Fourmy, D., Recht, M.I., Blanchard, S.C., Puglisi, J.D., 1996. Structure of the a site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science 274 (5291), 1367–1371.

Giacona, M.B., Papapanou, P.N., Lamster, I.B., Rong, L.L., D'Agati, V.D., Schmidt, A.M., et al., 2004. Porphyromonas gingivalis induces its uptake by human macrophages and promotes foam cell formation in vitro. FEMS Microbiol. Lett. 241 (1), 95–101.

Guidi-Rontani, C., 2002. The alveolar macrophage: the Trojan horse of Bacillus anthracis. Trends Microbiol. 10 (9), 405–409.

Guo, Y., Nguyen, K.A., Potempa, J., 2010. Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. Periodontology 2000 54 (1), 15–44.

Han, Y.W., Shi, W., Huang, G.T., Kinder Haake, S., Park, N.H., Kuramitsu, H., et al., 2000. Interactions between periodontal bacteria and human oral epithelial cells: fusobacterium nucleatum adheres to and invades epithelial cells. Infect. Immun. 68 (6), 3140–3146.

Haney, E.F., Nazmi, K., Bolscher, J.G., Vogel, H.J., 2012. Structural and biophysical characterization of an antimicrobial peptide chimera comprised of lactoferricin and lactoferrampin. Biochim. Biophys. Acta 1818 (3), 762–775.

Hooper, L.V., 2015. Epithelial cell contributions to intestinal immunity. Adv. Immunol. 126, 129–172.

Jiang, S.J., Xiao, X., Zheng, J., Lai, S., Yang, L., Li, J., et al., 2022. Antibacterial and antibiofilm activities of novel antimicrobial peptide DP7 against the periodontal pathogen Porphyromonas gingivalis. J. Appl. Microbiol. 133 (2), 1052–1062.

Kaneko, M., Emoto, Y., Emoto, M., 2016. A simple, reproducible, inexpensive, yet old-fashioned method for determining phagocytic and bactericidal activities of macrophages. Yonsei Med. J. 57 (2), 283–290.

Katz, J., Sambandam, V., Wu, J.H., Michalek, S.M., Balkovetz, D.F., 2000. Characterization of Porphyromonas gingivalis-induced degradation of epithelial cell junctional complexes. Infect. Immun. 68 (3), 1441–1449.

Krieger, J.N., Dickins, C.S., Rein, M.F., 1985. Use of a time-kill technique for susceptibility testing of trichomonas vaginalis. Antimicrob. Agents Chemother. 27 (3), 332–336.

Kulik, E.M., Lenkeit, K., Chenaux, S., Meyer, J., 2008. Antimicrobial susceptibility of periodontopathogenic bacteria. J. Antimicrob. Chemother. 61 (5), 1087–1091.

Lam, R.S., O'Brien-Simpson, N.M., Holden, J.A., Lenzo, J.C., Fong, S.B., Reynolds, E.C., 2016. Unprimed, M1 and M2 macrophages differentially interact with Porphyromonas gingivalis. PLoS One 11 (7), e0158629.

Lamont, R.J., Chan, A., Belton, C.M., Izutsu, K.T., Vasel, D., Weinberg, A., 1995. Porphyromonas gingivalis invasion of gingival epithelial cells. Infect. Immun. 63 (10), 3878–3885.

Larsen, T., 2002. Susceptibility of Porphyromonas gingivalis in biofilms to amoxicillin, doxycycline and metronidazole. Oral Microbiol. Immunol. 17 (5), 267–271.

Lorenzi, C., Lio, F., Mazzetti, V., Carosi, P., Lamelza, S., Pistoia, E.S., et al., 2024. Synergistic effect of metronidazole and chlorhexidine against Porphyromonas gingivalis in vitro study. Dent. J. (Basel) 12 (1).

Maezono, H., Noiri, Y., Asahi, Y., Yamaguchi, M., Yamamoto, R., Izutani, N., et al., 2011. Antibiofilm effects of azithromycin and erythromycin on Porphyromonas gingivalis. Antimicrob. Agents Chemother. 55 (12), 5887–5892.

Milazzo, I., Blandino, G., Caccamo, F., Musumeci, R., Nicoletti, G., Speciale, A., 2003. Faropenem, a new oral penem: antibacterial activity against selected anaerobic and fastidious periodontal isolates. J. Antimicrob. Chemother. 51 (3), 721–725.

Millan, D., Chiriboga, C., Patarroyo, M.A., Fontanilla, M.R., 2013. Enterococcus faecalis internalization in human umbilical vein endothelial cells (HUVEC). Microb. Pathog. 57, 62–69.

Nix, D.E., Tyrrell, R., Muller, M., 1995. Pharmacodynamics of metronidazole determined by a time-kill assay for trichomonas vaginalis. Antimicrob. Agents Chemother. 39 (8), 1848–1852.

Pankuch, G.A., Jacobs, M.R., Appelbaum, P.C., 1993. Susceptibilities of 428 gram-positive and -negative anaerobic bacteria to Bay y3118 compared with their susceptibilities to ciprofloxacin, clindamycin, metronidazole, piperacillin, piperacillin-tazobactam, and cefoxitin. Antimicrob. Agents Chemother. 37 (8), 1649–1654.

Phillips, I., Eykyn, S., King, B.A., Jenkins, C., Warren, C.A., Shannon, K.P., 1977. The in vitro antibacterial activity of nine aminoglycosides and spectinomycin on clinical isolates of common gram-negative bacteria. J. Antimicrob. Chemother. 3 (5), 403–410.

Rollin, G., Tan, X., Tros, F., Dupuis, M., Nassif, X., Charbit, A., et al., 2017. Intracellular survival of Staphylococcus aureus in endothelial cells: a matter of growth or persistence. Front. Microbiol. 8, 1354.

Ruangcharoen, S., Suwannarong, W., Lachica, M., Bolscher, J.G.M., Nazmi, K., Khunkitti, W., et al., 2017. Killing activity of LFchimera on periodontopathic bacteria and multispecies oral biofilm formation in vitro. World J. Microbiol. Biotechnol. 33 (9), 167.

Schlessinger, D., 1988. Failure of aminoglycoside antibiotics to kill anaerobic, low-pH, and resistant cultures. Clin. Microbiol. Rev. 1 (1), 54–59.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH image to ImageJ: 25 years of image analysis. Nat. Methods 9 (7), 671–675.

Sender, R., Fuchs, S., Milo, R., 2016. Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. Cell 164 (3), 337–340.

Soldati, K.R., Jiang, Y., Brandt, B.W., Exterkate, R.A.M., Buijs, M.J., Nazmi, K., et al., 2023. Differential modulation of saliva-derived microcosm biofilms by antimicrobial peptide LL-31 and D-LL-31. Pathogens 12 (11).

Soto, C., Rojas, V., Yanez, L., Hidalgo, A., Olivera, M., Pacheco, M., et al., 2022. Porphyromonas gingivalis-helicobacter pylori co-incubation enhances Porphyromonas gingivalis virulence and increases migration of infected human oral keratinocytes. J. Oral Microbiol. 14 (1), 2107691.

Taheri-Araghi, S., 2024. Synergistic action of antimicrobial peptides and antibiotics: current understanding and future directions. Front. Microbiol. 15, 1390765.

Van Dyck, K., Viela, F., Mathelie-Guinlet, M., Demuyser, L., Hauben, E., Jabra-Rizk, M. A., et al., 2020. Adhesion of Staphylococcus aureus to Candida albicans during co-infection promotes bacterial dissemination through the host immune response. Front. Cell. Infect. Microbiol. 10, 624839.

Vaudaux, P., Waldvogel, F.A., 1979. Gentamicin antibacterial activity in the presence of human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. 16 (6), 743–749.

Walter, C., Zahlten, J., Schmeck, B., Schaudinn, C., Hippenstiel, S., Frisch, E., et al., 2004. Porphyromonas gingivalis strain-dependent activation of human endothelial cells. Infect. Immun. 72 (10), 5910–5918.

Wang, H., Ai, L., Zhang, Y., Cheng, J., Yu, H., Li, C., et al., 2018. The effects of antimicrobial peptide Nal-P-113 on inhibiting periodontal pathogens and improving periodontal status. Biomed. Res. Int. 2018, 1805793.

Weisblum, B., Davies, J., 1968. Antibiotic inhibitors of the bacterial ribosome. Bacteriol. Rev. 32 (4 Pt 2), 493–528.

Werheim, E.R., Senior, K.G., Shaffer, C.A., Cuadra, G.A., 2020. Oral pathogen Porphyromonas gingivalis can escape phagocytosis of mammalian macrophages. Microorganisms 8 (9).

White, J.K., Muhammad, T., Alsheim, E., Mohanty, S., Blasi-Romero, A., Gunasekera, S., et al., 2022. A stable cyclized antimicrobial peptide derived from LL-37 with host immunomodulatory effects and activity against uropathogens. Cell. Mol. Life Sci. 79 (8), 411.

Wiegand, I., Hilpert, K., Hancock, R.E., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. 3 (2), 163–175.

Wongkaewkhiaw, S., Taweechaisupapong, S., Thanaviratananich, S., Bolscher, J.G.M., Nazmi, K., Anutrakunchai, C., et al., 2020. D-LL-31 enhances biofilm-eradicating effect of currently used antibiotics for chronic rhinosinusitis and its immunomodulatory activity on human lung epithelial cells. PLoS One 15 (12), e0243315.

Wright, T.L., Ellen, R.P., Lacroix, J.M., Sinnadurai, S., Mittelman, M.W., 1997. Effects of metronidazole on Porphyromonas gingivalis biofilms. J. Periodontal Res. 32 (5), 473–477.

Wu, X., Wang, Z., Li, X., Fan, Y., He, G., Wan, Y., et al., 2014. In vitro and in vivo activities of antimicrobial peptides developed using an amino acid-based activity prediction method. Antimicrob. Agents Chemother. 58 (9), 5342–5349.

Wu, X., Li, Z., Li, X., Tian, Y., Fan, Y., Yu, C., et al., 2017. Synergistic effects of antimicrobial peptide DP7 combined with antibiotics against multidrug-resistant bacteria. Drug Des. Dev. Ther. 11, 939–946.

Yu, L., Liu, H., E, L., Wu, X., Wang, D., 2009. Uptake of metronidazole by human gingival fibroblasts. J. Periodontol. 80 (6), 993–998.

Zheng, S., Yu, S., Fan, X., Zhang, Y., Sun, Y., Lin, L., et al., 2021. Porphyromonas gingivalis survival skills: Immune evasion. J. Periodontal Res. 56 (6), 1007–1018.