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Tunney, M. M., Payne, J. E., McGrath, S. J., Einarsson, G. G., Ingram, R. J., Gilpin, D. F., Juarez-Perez, V., & Elborn, J. S. (2018). Activity of hypothiocyanite and lactoferrin (ALX-009) against respiratory cystic fibrosis pathogens in sputum. *Journal of Antimicrobial Chemotherapy*. Advance online publication. <https://doi.org/10.1093/jac/dky357>

Published in:
Journal of Antimicrobial Chemotherapy

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

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Activity of hypothiocyanite and lactoferrin (ALX-009) against respiratory cystic fibrosis pathogens in sputum

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Running title: Antimicrobial activity of ALX-009

Keywords: ALX-009, OSCN⁻, Lactoferrin, sputum, Cystic Fibrosis, *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex

OBJECTIVES: To determine the antimicrobial activity of ALX-009, a combination of bovine lactoferrin and hypothiocyanite, in sputum against *P. aeruginosa* and *Burkholderia cepacia* complex (Bcc), key pathogens causing infection in the lungs of Cystic Fibrosis (CF) patients.

METHODS: The antimicrobial activity of ALX-009 against clinical respiratory *P. aeruginosa* isolates was determined by time-kill. Sputum from CF patients was treated with ALX-009, either alone or in combination with tobramycin, and the effect on *P. aeruginosa*, Bcc and total sputum density determined.

RESULTS: By time-kill, ALX-009 was bactericidal at 24 hours against 4/4 *P. aeruginosa* isolates under aerobic conditions and 3/4 isolates under anaerobic conditions. ALX-009 was also bactericidal against *P. aeruginosa* in sputum samples at 6 (n=22/24 samples) and 24 hours (n=14/24 samples) and demonstrated significantly greater activity than tobramycin at both timepoints. Activity against Bcc in sputum samples (n=9) was also demonstrated but the magnitude of change in Bcc density was less than for *P. aeruginosa*. To determine the effect of treating sputum with two doses of ALX-009, similar to current regimens for inhaled antibiotics, aliquots of a further 10 *P. aeruginosa* positive sputum samples were treated with one (t=0 h) or two doses (t=0 h, t=12 h) of ALX-009; treatment with 2 doses resulted in bactericidal activity in 7/10 samples at 34 hours compared with only 3/10 samples when treatment was with one dose.

CONCLUSIONS: ALX-009 demonstrates promise as a novel antimicrobial that could be used to decrease *P. aeruginosa* density in the lungs of people with CF.

Cystic Fibrosis (CF) is characterised by chronic polymicrobial airway infection and inflammation which is the major cause of morbidity and mortality.¹ Although *Pseudomonas aeruginosa* is the most frequently isolated CF respiratory pathogen,² infection with other bacteria including *Staphylococcus aureus*, *Burkholderia cepacia* complex (Bcc), *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* also occurs.² Many of these bacteria are constitutively resistant to a range of antibiotics. Moreover, frequent use of antibiotics for suppression of bacterial growth and treatment of exacerbations results in further development of resistance.²⁻⁵ In a recent study in Northern Europe, 60% of CF *P. aeruginosa* isolates were multidrug resistant (MDR) with resistance to penicillins and cephalosporins most common.⁶

There is a clear unmet need for novel antimicrobial agents to treat respiratory infection, particularly those caused by *P. aeruginosa* and Bcc, in CF patients. One such antimicrobial is ALX-009, an investigational inhaled product in development by Alaxia (Lyon, France) for treatment of bacterial respiratory infections. ALX-009 is a combination of bovine lactoferrin (bLF) and hypothiocyanite (OSCN⁻). Lactoferrin is an antimicrobial protein found in airway secretions;⁷ however, it has been detected at low levels in the CF lung and levels are further depleted in patients colonized with *P. aeruginosa* due to degradation by proteases.⁸ Hypothiocyanite is a bactericidal agent that is naturally produced as part of the oxidative lactoperoxidase-hydrogen peroxide-thiocyanate system.⁹ In CF, this system is defective and the bactericidal OSCN⁻ cannot form.¹⁰ ALX-009 has been recently shown to have *in vitro* bactericidal activity against a range of CF pathogens including *P. aeruginosa* (n=52), MDR *P. aeruginosa* (n=20) and *Burkholderia spp.* (n=165).¹¹ Furthermore, ALX-109 (8,000 mg/L apo-bLF and 100 µM OSCN⁻) prevents biofilm formation by *P. aeruginosa* on airway epithelial cells.¹² Moreover, although *in vitro* studies have reported contradictory results with respect to the cytotoxicity of lactoferrin,¹²⁻¹⁴ a recent safety study in which ALX-009 was administered

daily to rats and dogs for up to 6 and 9 months, respectively, reported no histological signs indicative of cell death.¹⁵

In this study, we determined the activity of ALX-009 against clinical respiratory *P. aeruginosa* isolates grown planktonically under aerobic and anaerobic conditions. To determine both efficacy under physiologically relevant conditions and potential synergy with tobramycin, an antibiotic currently used to treat lung infection in CF patients, we treated sputum from CF patients with ALX-009, alone and in combination with tobramycin, and determined the effect on bacterial density of *P. aeruginosa*, Bcc and total sputum bacterial density.

Materials and methods

Materials

Hypothiocyanite (OSCN^-) and lactoferrin solutions were prepared, according to an Alaxia SAS standard operating procedure, and mixed to give a final concentration of ALX-009 (hypothiocyanite, 130 mg/L; lactoferrin, 8, 000 mg/L) which corresponded to the MIC₉₀ for a large collection of clinical isolates previously tested.^{11, 16} Tobramycin sulphate (USP grade), obtained from Chongqing Biochem, China, was dissolved in sterile water to achieve a stock solution of 10, 000mg/L. Glucose-6-phosphate (G6P) and potassium nitrate (KNO_3) were both purchased from Sigma-Aldrich (Gillingham, UK). *P. aeruginosa* selective agar (PASA) and *B. cepacia* specific agar (BCSA) were purchased from Fannin Healthcare (Leopardstown, Ireland). Mueller Hinton agar (MHA, Oxoid Ltd, Basingstoke, UK) and Mueller Hinton broth (MHB; Oxoid Ltd, Basingstoke, UK) were supplemented with 1% (w/v) potassium nitrate when testing *P. aeruginosa* to act as the terminal electron receptor and allow for anaerobic respiration. G6P was added to the media at a final concentration of 25mg/L for all time-kill assays.

Bacterial isolates

P. aeruginosa isolates (B023 V4S2C, B002 V1S1A, B060 V1S1K and B008 V1S1C) used in the study were cultured from the sputum of four different CF patients at the Adult CF Centre, Belfast Health and Social Care Trust. Isolates were cultured on Supplemented Brucella Blood Agar (SBBA) at 37°C under aerobic conditions for 24 hours prior to use.

Time-kill studies

Time-kill experiments were performed in MHB according to CLSI standards¹⁷ to determine the activity of ALX-009 against *P. aeruginosa* isolates. Bacterial cultures, at a final inoculum of approximately 5×10^5 cfu/ml, were incubated with ALX-009 at 37°C under aerobic or anaerobic conditions. Killing activity was assessed at 0, 1, 3, 6 and 24 h by carrying out serial dilutions (10^{-1} to 10^{-6}) in quarter strength Ringers solution (QSRS) followed by enumerating on MHA. A no drug control was run in each assay. Bactericidal activity was defined as a ≥ 3 log₁₀ reduction in colony forming units (cfu/ml) in the original inoculum.¹⁸

Sputum sample collection and processing

Anonymised sputum samples, which would have been disposed of, were collected from CF patients during physiotherapy treatment at the adult CF Centre, Belfast Health and Social Care Trust. Permission to use these anonymised sputum samples was given by the Director of R&D in the Belfast Health and Social Care Trust. Immediately upon sample receipt, an aliquot was homogenised by repeated passage through a 1 ml syringe (BD Plastipak, Franklin Lakes, USA) without a needle. Aliquots (100 µl) were spread plated on PASA or BCSA, for selection of *P. aeruginosa* and Bcc, respectively, and blood agar plates to confirm bacterial growth in the sample. Plates were incubated until growth was visible for a maximum of 3 days. Growth of

P. aeruginosa or Bcc on selective plates was confirmed by 16S rRNA sequencing as described previously.¹⁹ Susceptibility of *P. aeruginosa* isolates, cultured from these samples, to tobramycin was determined by Etest[®] according to the manufacturer's instructions. Additional aliquots of each sputum sample were stored at -80°C until required for further use.

Antimicrobial activity of ALX-009 against P. aeruginosa and Bcc in CF sputum

Sputum samples (n=24, *P. aeruginosa* positive; n=9, Bcc positive) were allowed to thaw on ice. No sputum samples were positive for both *P. aeruginosa* and Bcc. Sputum plugs were selected and homogenised as described above. Aliquots (0.5ml) were added to tubes containing 4.5ml of ALX-009, tobramycin, ALX-009 and tobramycin or Phosphate buffered saline (PBS; control). Tobramycin was used at a final concentration of 8 mg/L with 4 µl of tobramycin stock solution added to either sterile PBS or ALX-009 to determine activity of tobramycin alone and in combination with ALX-009, respectively. Samples were vortex mixed and incubated at 37°C for 24 hours under aerobic conditions. At 0, 6 and 24 hours, aliquots (100µl), were removed, serially diluted in QSRS solution and plated on both non-selective blood agar and PASA or BCSA plates. Drug carry-over was minimised by carrying out serial dilutions (10^{-1} to 10^{-5}) in sterile QSRS. Following overnight incubation in air, *P. aeruginosa*/Bcc density and total bacterial density were determined with *P. aeruginosa* and Bcc identified as described above. A reduction, by any of the agents/combinations under test, of the original bacterial density by $\geq 3 \log_{10}$ cfu/g sputum was considered bactericidal. Synergy was defined, according to CLSI guidelines, as a $\geq 2 \log_{10}$ decrease in bacterial density at 24 hours by the combination compared with that by the most active single agent and as a $\geq 2 \log_{10}$ decrease in bacterial density compared with the starting bacterial density.^{17, 18} Results for time-kill assays are expressed as median cfu/g sputum with range.

Effect of treatment with two doses of ALX-009 on microbial load in CF sputum

In clinical practice, it is likely that ALX-009 would be administered twice daily in a similar dosing regimen to other currently available inhaled antibiotics such as tobramycin and colistin. To determine the effect of treating sputum with a 2nd dose of ALX-009, aliquots from an additional 10 *P. aeruginosa* positive sputum samples were treated with one (t=0 h) or two doses (t=0 h, t=12 h) of ALX-009 or control. Following addition of test agents, aliquots (100µl), were removed at 0, 6 and 24 hours after the first dose and at 0, 6 and 22 hours after the second dose which corresponded to 12, 18 and 34 hours after initial treatment. Aliquots were serially diluted, plated and *P. aeruginosa* and total bacterial density determined as described above.

Statistical analysis

Comparison of *P. aeruginosa*, Bcc and total sputum bacterial density between treatment groups was performed by the Friedman test with post hoc tests performed using the Wilcoxon signed-rank test with Bonferroni adjustment for multiple groups applied. Spearman's rank was used to determine if there was a correlation between either initial *P. aeruginosa* sputum bacterial density or tobramycin MIC for *P. aeruginosa* isolates and *P. aeruginosa* density following treatment for 24 hours with a single dose of ALX-009 or tobramycin.

Results

Antimicrobial activity of ALX-009 against clinical *P. aeruginosa* isolates under aerobic and anaerobic conditions

Bactericidal activity of ALX-009 was dependent on the *P. aeruginosa* isolates tested (Fig. 1). For two of the isolates, killing was rapid; bacterial numbers decreased to below the detectable

limit after 2 hours with no further growth detected at subsequent timepoints (Supplementary Fig. 1). However, for the other two isolates (Supplementary Fig. 1), although initial killing was rapid with bacterial numbers decreasing to below the detectable limit after 2 hours, further growth was apparent at 24 hours. At 24 hours, ALX-009 demonstrated bactericidal activity against all 4 isolates under aerobic conditions and 3/4 isolates under anaerobic conditions.

Antimicrobial activity of ALX-009 against P. aeruginosa in CF sputum

ALX-009 demonstrated bactericidal activity against *P. aeruginosa* in sputum samples (n=24) at 6 and 24 hours (Fig. 2a). At 6 hours, bactericidal activity was apparent in 22/24 samples with *P. aeruginosa* sputum density below the detectable limit in 12/24 samples. At 24 hours, bactericidal activity was apparent for 14/24 samples with *P. aeruginosa* sputum density below the detectable limit in 10/24 samples. There were significant differences ($p<0.001$) in *P. aeruginosa* sputum density between the four treatments at 6 and 24 hours. At 6 hours, the median *P. aeruginosa* sputum density was significantly lower ($p<0.001$) in ALX-009 (5.5×10^0 cfu/g sputum [range 0 - 2.4×10^4 cfu/g sputum]) treated versus control (1.08×10^6 cfu/g sputum [range 190 – 1.09×10^8 cfu/g sputum]) samples (Fig. 2b). At 24 hours, the median *P. aeruginosa* sputum density was also significantly lower ($p<0.001$) in ALX-009 (3.7×10^2 cfu/g sputum [range 0 – 1.43×10^8 cfu/g sputum]) treated versus control samples (2.35×10^7 cfu/g sputum [range 0 – 2.09×10^8 cfu/g sputum]) (Fig. 2C). ALX-009 also demonstrated significantly greater bactericidal activity than tobramycin at both 6 ($p<0.001$) and 24 ($p=0.004$) hours. Tobramycin demonstrated bactericidal activity against *P. aeruginosa* in 3/24 samples and bacteriostatic activity against *P. aeruginosa* in 2/24 samples at 24 hours; however, *P. aeruginosa* density was only reduced below the detectable limit in one sample.

Changes in *P. aeruginosa* density following treatment with ALX-009 reflected initial *P. aeruginosa* density in the sample. In the 10 samples, in which *P. aeruginosa* density was

reduced below the detectable limit at 24 hours, initial *P. aeruginosa* density was approximately 10^4 CFU/g sputum (median, 1.17×10^4 ; range, 4×10^3 - 1.45×10^6 cfu/g sputum). In contrast, for the remaining 14 samples, in which *P. aeruginosa* was detected at 24 hours, initial *P. aeruginosa* density was higher at approximately 10^6 cfu/g sputum (median, 1.05×10^6 ; range, 3.3×10^4 - 4.20×10^7 cfu/g sputum). There was a significant correlation (Spearman's $\rho = 0.6128$, $p = 0.0015$) between the initial *P. aeruginosa* density in sputum and the *P. aeruginosa* density in sputum at 24 hours following treatment with ALX-009.

The susceptibility to tobramycin of the most prevalent *P. aeruginosa* morphotype in each of the 24 *P. aeruginosa* positive samples was determined; 10 isolates were resistant to tobramycin with the remaining 14 isolates sensitive (Supplementary Table 1). There was no correlation between tobramycin MIC and *P. aeruginosa* density at 24 hours following treatment with either tobramycin (Spearman's $\rho = 0.353$, $p = 0.091$) or ALX-009 (Spearman's $\rho = -0.03$, $p = 0.8879$).

Given the bactericidal activity of ALX-009 against *P. aeruginosa* in sputum, in the majority of samples, it was not possible to determine if there was a synergistic effect when ALX-009 was combined with tobramycin. However, synergy was apparent for 4 samples at 24 hours (Supplementary Fig. 2).

The effect of ALX-009 on total sputum bacterial density was less than that observed for *P. aeruginosa*. However, there were statistically significant differences ($p < 0.001$) between the four test conditions at 6 (Fig. 3A) and 24 (Fig. 3B) hours. Post hoc tests revealed that there were significant differences in the median total density when samples were treated with ALX-009 versus both control and tobramycin at both timepoints.

Antimicrobial activity of ALX-009 against Bcc in CF sputum

ALX-009 demonstrated activity against Bcc (*B. cenocepacia*, n=5; *B. multivorans*, n=4) in sputum samples (Fig. 4a.) but the magnitude of change in Bcc density following treatment was less than that for *P. aeruginosa*. The median Bcc density was significantly lower in ALX-009 (6h, 6.80×10^4 cfu/g sputum; 24h, 1.77×10^5 cfu/g sputum) treated versus control (6h, 1.21×10^7 cfu/g sputum; 2.15 $\times 10^7$ cfu/g sputum) samples; however, Bcc density was not reduced below the detectable limit in any sample at either time-point. Tobramycin had no bactericidal activity against Bcc and no synergy was apparent between ALX-009 and tobramycin for the 5 samples in which both agents were tested.

The effect of ALX-009 on total bacterial density in Bcc positive samples was also determined (Fig. 4b). The median total density following treatment with ALX-009 (6h, 7.20×10^5 cfu/g sputum; 24h, 1.04×10^7 cfu/g sputum) was significantly less than the untreated control (6h, 3.70×10^7 cfu/g sputum; 24h, 1.04×10^7 cfu/g sputum). In contrast, tobramycin had no effect on total bacterial density in Bcc positive sputum samples.

Effect of treatment with a second dose of ALX-009 on bacterial density in CF sputum

To determine the effect of treating sputum with a second dose of ALX-009, aliquots of a further 10 *P. aeruginosa* positive sputum samples were treated with one (t=0 h) or two doses (t=0 h, t=12 h) of ALX-009; treatment with 2 doses resulted in bactericidal activity in 7/10 samples at 34 hours compared with only 3/10 samples with one dose (Fig. 5A). There were significant differences ($p < 0.001$) between the three treatment groups at 12, 18, 24 and 34 hours (Supplementary Fig. 3). There were also significant differences ($p < 0.001$) in total sputum bacterial density between the three treatment groups at 12, 18, 24 and 34 hours (Fig. 5B, Supplementary Fig. 4). For both *P. aeruginosa* (Supplementary Fig. 3) and total

(Supplementary Fig. 4) sputum density, post hoc tests demonstrated significant differences between control and both single and two doses and also between single dose and two doses.

Discussion

ALX-009, a novel antimicrobial compound composed of hypothiocyanite and bovine lactoferrin, was granted orphan drug status in 2009 and is currently under evaluation for aerosol administration in people with CF with chronic respiratory infection. This study investigated the antimicrobial activity of ALX-009, alone and in combination with tobramycin, against CF respiratory pathogens in sputum.

Given that sputum within the lungs of CF patients contains anaerobic microenvironments²⁰ and that hypoxic environments impact the activity of some classes of antibiotics,²¹⁻²³ we initially examined activity under both aerobic and anaerobic conditions. In time-kill assays, ALX-009 demonstrated bactericidal activity against *P. aeruginosa* isolates under both aerobic and anaerobic conditions. These results are consistent with previous studies that reported inhibitory and bactericidal activity of ALX-009, using broth microdilution and time-kill assays, respectively.^{11, 16} ALX-009 maintained activity against *P. aeruginosa* under anaerobic conditions suggesting that antimicrobial activity would not be decreased under anaerobic conditions within the CF lung. This contrasts with the activity of some antibiotics currently used to treat CF lung infection such as tobramycin, amikacin and aztreonam where MICs for *P. aeruginosa* are significantly higher under anaerobic conditions.²¹⁻²⁴

To determine activity under conditions which are more physiologically relevant to *in vivo* conditions in the CF lung, we determined activity against key respiratory pathogens in CF sputum. CF sputum contains DNA, filamentous actin, mucin, bacteria and cell debris from bacterial, inflammatory and epithelial cells²⁵ which can all adversely affect the activity of

antimicrobials.²⁶ Furthermore, it is widely recognised that bacteria grow in complex polymicrobial biofilms within CF sputum and that this causes increased resistance to antibiotics.²⁷⁻²⁹ Stability of hypothiocyanite and lactoferrin in sputum is unknown. Moreover, due to the complexity of the sputum matrix, specific and well adapted analytical methods are required to measure both compounds and such methods are not presently available. Hypothiocyanite has a short half-life of about 1 hour in buffered solutions³⁰ and therefore drug carry-over is not of concern. Lactoferrin has a longer half-life and potential drug carry-over was accounted for by immediately carrying out serial dilutions in QSRS on removal of the sample aliquot. Treatment of sputum from CF patients with ALX-009 resulted in a significant decrease in the bacterial density of both *P. aeruginosa* and Bcc. The magnitude of effect was greater for *P. aeruginosa* than Bcc which is not unexpected given the inherent antimicrobial resistance of Bcc.³¹ These findings support the results of a previous study which reported that ALX-109 reduced *P. aeruginosa* (PAO1) biofilm formation by 0.7 log units, corresponding to a killing of ~60% of the bacteria compared with untreated control.¹² We also demonstrated synergy between ALX-009 and tobramycin against *P. aeruginosa* in sputum; however, this was only apparent in a small number of sputum samples due to the excellent bactericidal activity of ALX-009. These results support previous findings reporting an additive effect for tobramycin or aztreonam in combination with ALX-109 when disrupting established *P. aeruginosa* biofilms¹² and suggest that there could be benefit to administering ALX-009 in combination with antibiotics.

Changes in *P. aeruginosa* density in sputum, following a single treatment with ALX-009, reflected the initial *P. aeruginosa* density in the sample. To determine if differences in killing rates could be due to presence of antibiotic resistant *P. aeruginosa* and not an inoculum effect, we determined the susceptibility of *P. aeruginosa* from each sputum sample to tobramycin. There was no correlation between tobramycin MIC and *P. aeruginosa* density following

treatment with ALX-009 for 24 hours with ALX-009 demonstrating killing of both tobramycin susceptible and resistant *P. aeruginosa*. This suggests that cross resistance to ALX-009 from pre-existing antibiotic resistance mechanisms in *P. aeruginosa* is unlikely.

Given that changes in *P. aeruginosa* density in sputum, following a single treatment with ALX-009, reflected initial *P. aeruginosa* density in the sample, we hypothesized that repeated treatment with ALX-009 could further reduce *P. aeruginosa* airway bacterial density. Therefore, sputum samples, in which *P. aeruginosa* was present in a high load, were treated with two doses of ALX-009 12 hours apart to mimic the time-frame routinely used for administration of currently inhaled antibiotics; addition of a 2nd dose resulted in enhanced bactericidal activity against *P. aeruginosa*. However, further work is necessary to evaluate the effect of prolonged treatment with ALX-009 on *P. aeruginosa* airway bacterial density and the potential for development of resistance to ALX-009 and cross-resistance to other antimicrobials.

Extended culture and molecular detection methods have demonstrated a complex and diverse microbiota in the CF airways³²⁻³⁴ with loss of diversity associated with increasing age, reduced lung function, and disease progression.³⁵⁻³⁷ Therefore, agents which directly target CF pathogens, such as *P. aeruginosa*, without having bactericidal activity against other members of the airway microbiota could be beneficial. In the present study, there was a significant decrease in the total sputum bacterial density following both a single dose and two doses of ALX-009. However, the magnitude of this decrease was less than that apparent for *P. aeruginosa* and the total bacterial density increased once treatment with ALX-009 was discontinued. This suggests that treatment with ALX-009 may have less effect on total bacterial burden and may potentially have less effect on microbiota composition and structure. Detailed analysis of samples from future clinical studies would be required to better define the effect of

treatment on airway community composition and structure and to determine if the community fully recovers following completion of treatment.

There are a number of limitations to this study. Firstly, it would have been interesting to determine whether the use of a higher concentration of OSCN⁻ or bLF in the combination would have demonstrated greater antimicrobial activity against Bcc species. The concentrations of OSCN⁻ and bLF used corresponded to the MIC₉₀ for a large collection of clinical isolates previously tested.^{11, 16} As clinical doses have not yet been defined, there is the potential to increase the dose of both OSCN⁻ and bLF. Secondly, the number of Bcc positive sputum samples available, and the volume of sputum received in the samples were small. To better determine the activity of ALX-009 against Bcc a larger number of samples would be required, that are preferably greater in volume to enable determination of possible synergy with antibiotics. Thirdly, as sputum samples were exposed to a maximum of two doses, we were not able to determine if isolates in sputum are likely to determine resistance to ALX-009. However, if ALX-009 was to be used clinically, it would be important to determine if resistance would develop with prolonged use. Furthermore, given that ALX-009 demonstrated bactericidal activity against *P. aeruginosa* under both aerobic and anaerobic conditions, it would have also been interesting and clinically relevant to determine activity in sputum under anaerobic conditions. However, this was not feasible due to the number and volume of sputum samples available for testing.

In conclusion, this study has demonstrated the excellent antimicrobial activity of ALX-009 against *P. aeruginosa* isolates grown under both aerobic and anaerobic conditions. Moreover, it also clearly showed the bactericidal activity of ALX-009 against *P. aeruginosa* in sputum from CF patients. Therefore, ALX-009 demonstrates promise as a novel antimicrobial that could be used to treat multidrug resistant infections caused by *P. aeruginosa* in CF.

Funding

This work was supported by Alaxia, Lyon, France. J.E.P. was supported by a Department of Employment and Learning, Northern Ireland Studentship.

Transparency declaration

VJP works as a consultant for Alaxia SAS. The other authors do not declare any conflict of interest.

Author contributions

M.M.T., D.F.G. J.S.E. and V.J.P. conceived and designed research; J.E.P. and S.J.McG. performed research; M.M.T., J.E.P. and G.G.E. analyzed data; M.M.T., J.E.P., V.J.P. and J.S.E. wrote the paper; G.G.E., D.F.G. and R.J.I. review and revision of paper.

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FIG. 1

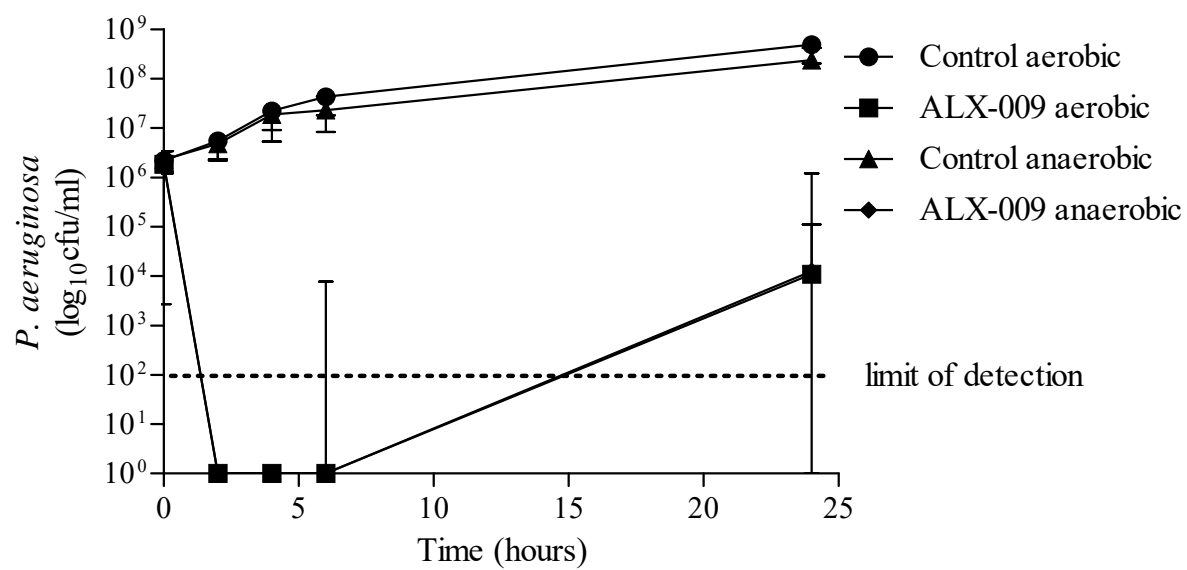
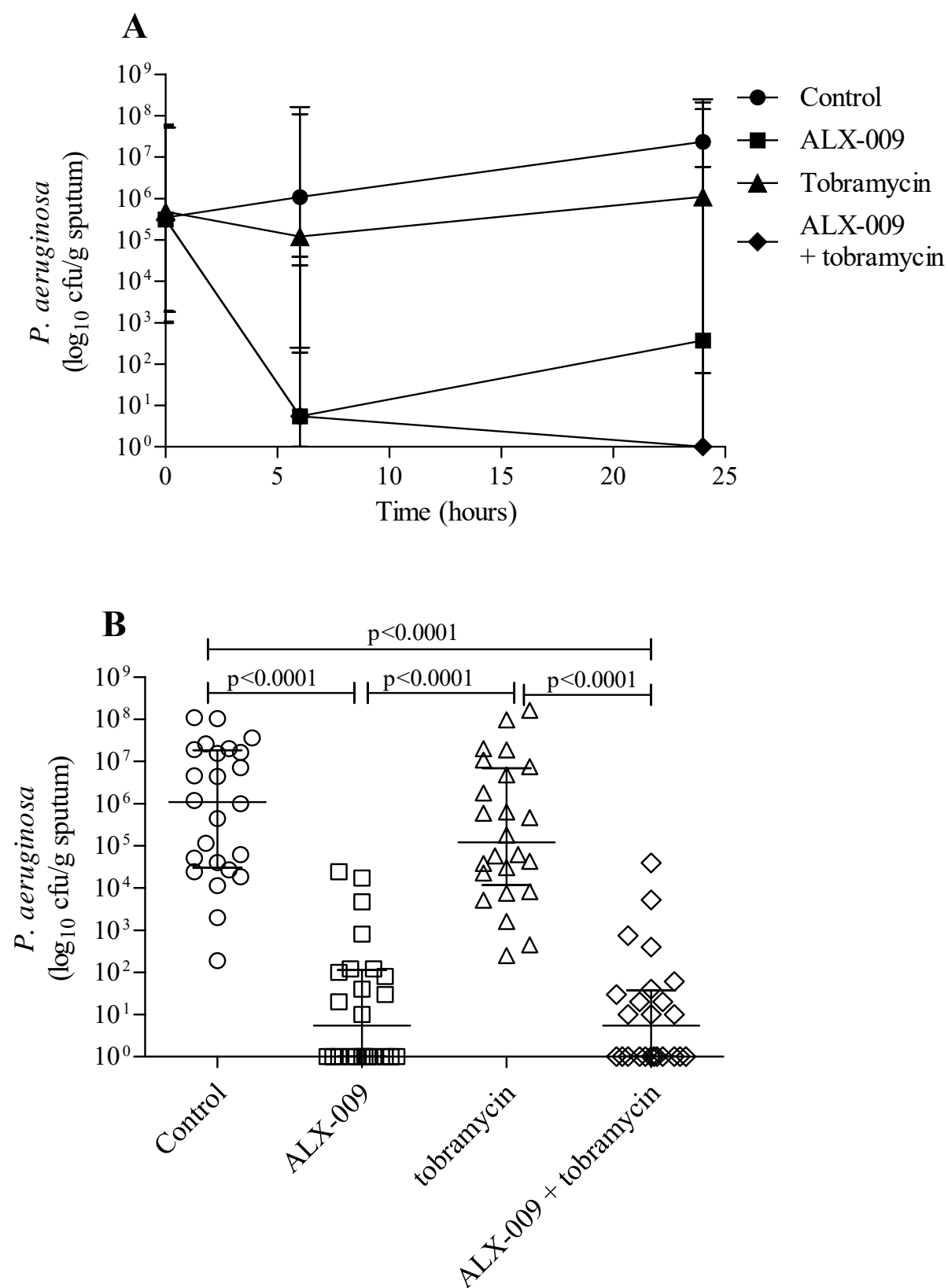


Fig. 1. Antimicrobial activity of ALX-009 against *P. aeruginosa* isolates (n=4) under aerobic and anaerobic conditions plotted as median cfu/ml with range.

FIG. 2



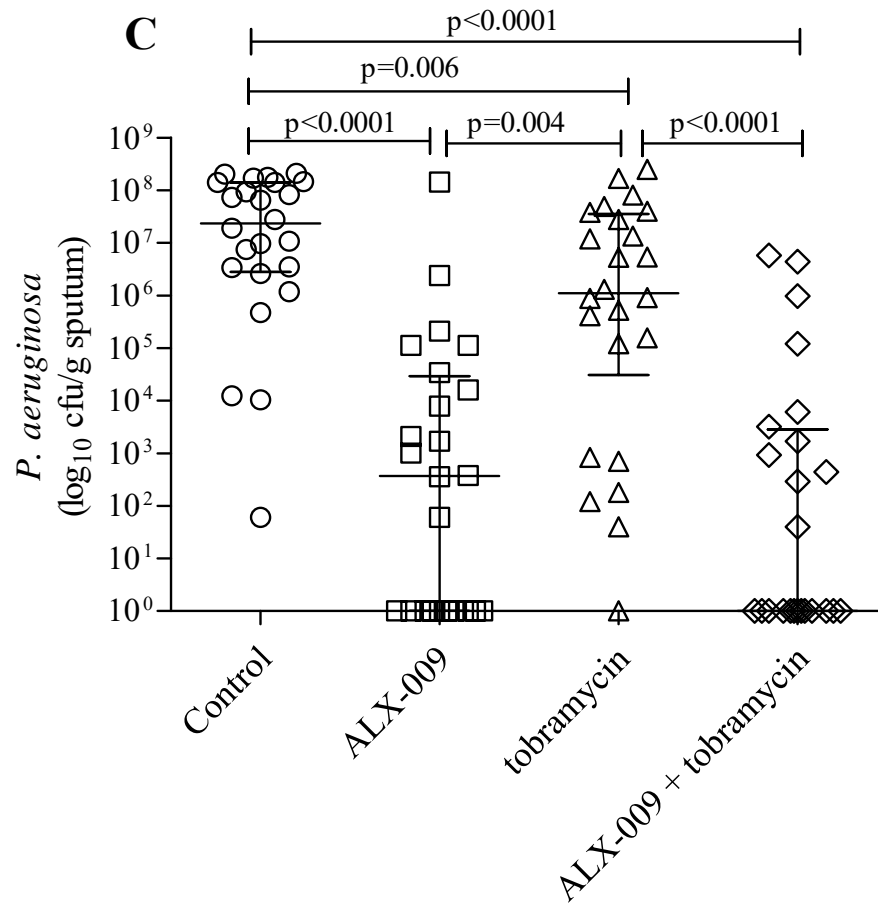


Fig. 2. A) Antimicrobial activity of ALX-009 against *P. aeruginosa* in sputum samples (n=24) over time presented as median total viable count with range. Total viable count of *P. aeruginosa* at (B) 6 hours and (C) 24 hours with median and interquartile range.

FIG.3.

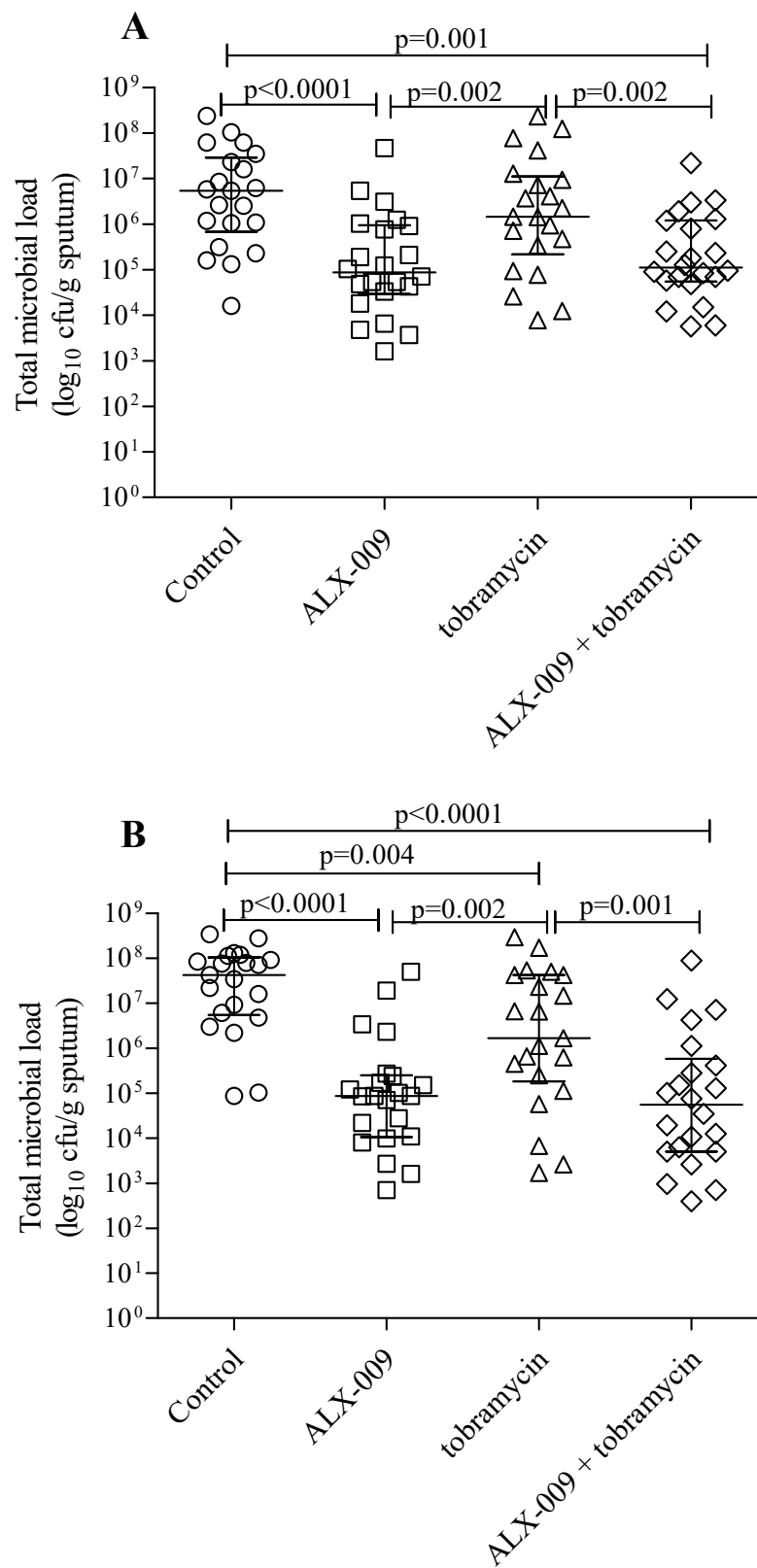


Fig. 3. Total microbial load at (A) 6 hours and (B) 24 hours with median and interquartile range. Significance was calculated using the Wilcoxon signed-rank test with Bonferroni adjustment for multiple comparisons.

FIG. 4

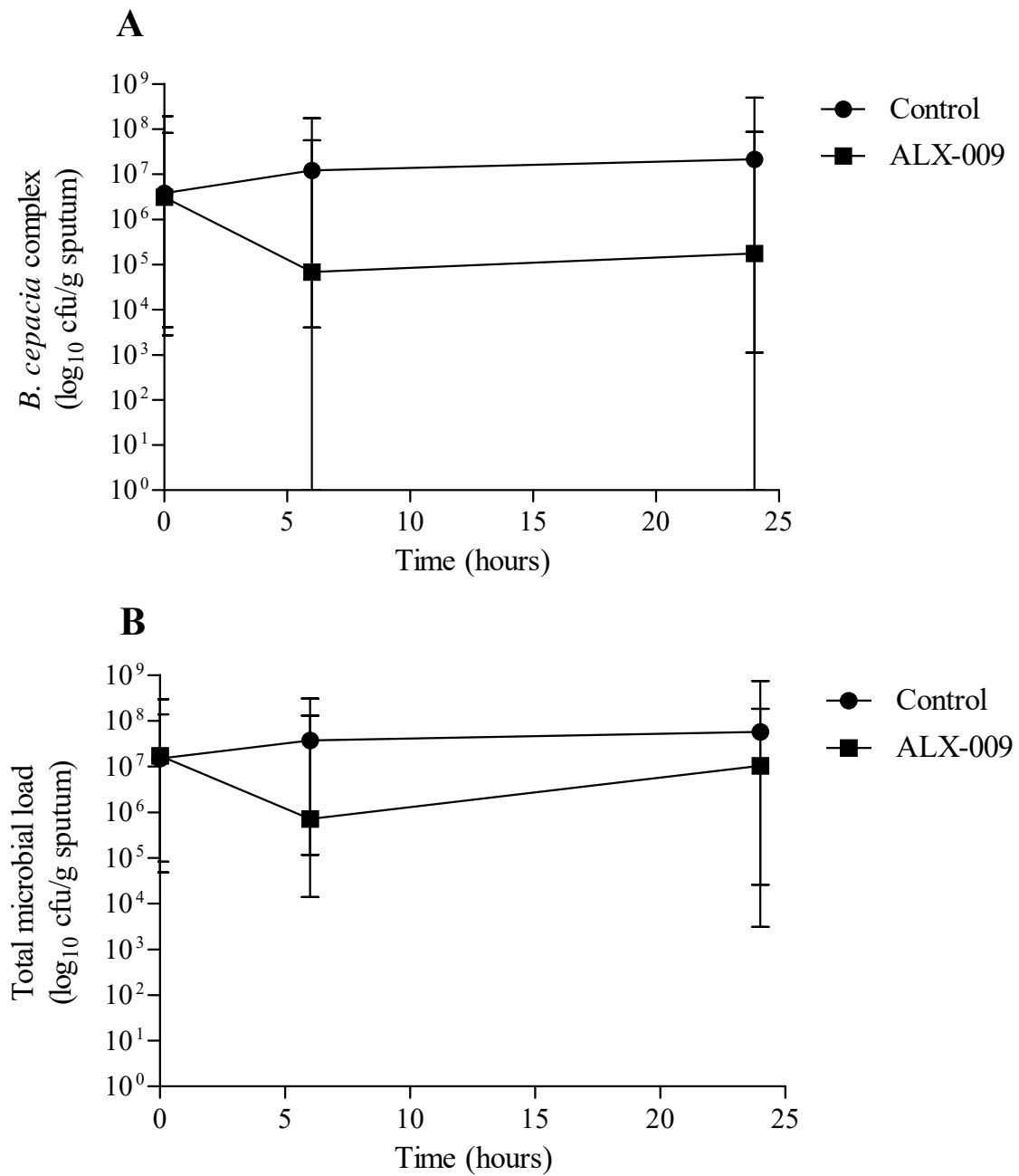


Fig.4. Antimicrobial activity of ALX-009 against (A) *B. cepacia* complex and (B) total microbial load in sputum samples (n=9) over time. Data presented as median total viable count with range.

FIG. 5

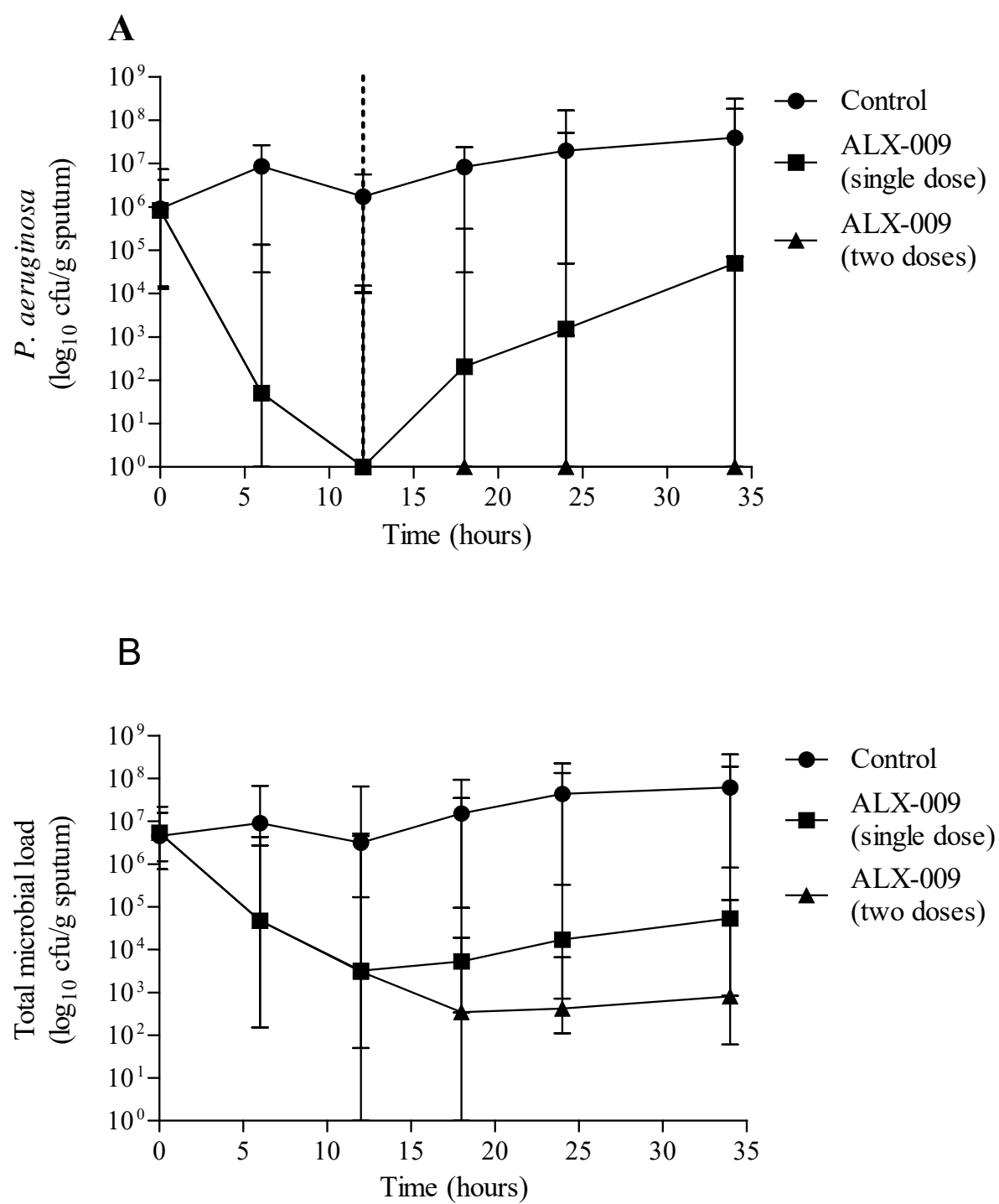


Fig. 5. Time-kill assay demonstrating the effect of two doses of ALX-009 administered at 12 hours (dotted line) against (A) *P. aeruginosa* and (B) total microbial load in sputum samples (n=10). Results are presented as the median cfu/g sputum with range.