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Modulation of the activity of moxifloxacin and solithromycin in an *in vitro* pharmacodynamic model of *Streptococcus pneumoniae* naive and induced biofilms

Nathalie M. Vandevelde[1], Paul M. Tulkens[1], Giulio G. Muccioli[2] and Françoise Van Bambeke[1]*

[1]*Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium;*
[2]*Bioanalysis and Pharmacology of Bioactive Lipids, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium*

*Corresponding author. Tel: +32-2-7647378; E-mail: francoise.vanbambeke@uclouvain.be

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Objectives: Bacterial biofilms developing in the bronchial tree of patients experiencing acute exacerbations of chronic bronchitis (AECBs) are suggested to cause relapses and recurrences of the disease because the matrix barrier impairs antibiotic access to the offending organisms. We examined whether bronchodilators could modulate pneumococcal biofilm development and antibiotic action using an *in vitro* model.

Methods: *Streptococcus pneumoniae* strains from patients hospitalized for AECBs and two reference strains (ATCC 49619 and R6) were screened for biofilm formation (multi-well plates; 2–11 days of growth). Ipratropium and salbutamol (alone or in combination) were added at concentrations of 1.45 and 7.25 mg/L, respectively (mimicking those in the bronchial tree), and their effects were measured on biofilm formation and modulation of the activity of antibiotics [full antibiotic concentration-dependent effects (pharmacodynamic model)] with a focus on moxifloxacin and solithromycin. Bacterial viability and biomass were measured by the reduction of resazurin and crystal violet staining, respectively. Release of sialic acid (from biofilm) and neuraminidase activity were measured using enzymatic and HPLC–MS detection of sialic acid.

Results: All clinical isolates produced biofilms, but with fast disassembly if from patients who had received muscarinic antagonists. Ipratropium caused: (i) reduced biomass formation and faster biofilm disassembly with free sialic acid release; and (ii) a marked improvement of antibiotic activity (bacterial killing and biomass reduction). Salbutamol stimulated neuraminidase activity associated with improved antibiotic killing activity (reversed by zanamivir) but modest biomass reduction.

Conclusions: Ipratropium and, to a lesser extent, salbutamol may cooperate with antibiotics for bacterial clearance and disassembly of pneumococcal biofilms.

Keywords: sialic acid, neuraminidase, zanamivir, viability, biomass, crystal violet, resazurin

Introduction

Streptococcus pneumoniae is one of the main pathogens associated with acute exacerbations of chronic bronchitis (AECBs).[1,2] Its capacity to form biofilms favours its persistence in the airways[3] and is likely to contribute to chronic colonization[4,5] leading to recurrences and/or relapses.[6] Within biofilms, bacteria are embedded in an extracellular matrix made of polymeric substances that creates a diffusion barrier to antibiotics, thereby reducing their activity.[4,7,8] For these reasons, destructuring the biofilm matrix could be an appealing strategy to improve antibiotic effectiveness,[9–12] even though the mechanism of action of the substances used in this context may be unrelated to their expected primary pharmacological activity.[13]

Patients with COPD usually receive bronchodilators. Ipratropium (a muscarinic antagonist that does not inhibit mucociliary clearance from bronchi)[14] and salbutamol (a selective β_2-agonist, with minimal untoward effects on cardiac rhythm, especially if given by inhalation)[15] are both recommended as short-acting bronchodilators for use in first-line therapy.[16] Beyond their primary and well-established relaxing effects on bronchial smooth muscles,[17] we wondered whether these drugs could also act by modifying the development of pneumococcal biofilms and their susceptibility to antibiotics, but could find no relevant published data. We therefore decided to address this issue directly by taking advantage of the recent development of an *in vitro* pharmacodynamic model of *S. pneumoniae* biofilms in which the activity of antibiotics against biofilms can be quantified with respect to both

bacterial viability and biomass.[18] This model enabled us to observe that *S. pneumoniae* isolates from AECB patients easily produce both naive and induced biofilms (reflecting primary infection and secondary colonization, respectively).[19]

In the present study, we first screened clinical isolates from the sputum of patients admitted to hospital with a confirmed diagnosis of AECBs for their capacity to produce biofilm *in vitro*.[18] Having observed no significant differences in biofilm formation between these clinical isolates and two reference strains, we focused on these reference strains and one selected clinical isolate to investigate the influence exerted by ipratropium and salbutamol on biofilm growth. For antibiotics, we first selected amoxicillin and clarithromycin as representatives of β-lactams and macrolides, respectively, and commonly recommended for patients with AECBs.[16] We then moved to moxifloxacin (because of its reported higher efficacy in the treatment of AECBs compared with other antibiotics)[20,21] and to solithromycin, a new fluoroketolide[22,23] active against contemporary macrolide-resistant strains[19,24] and currently in Phase III clinical development for the treatment of lower respiratory tract infections, in comparison with moxifloxacin.[25] These two antibiotics have also been shown to be the most effective within their respective class in the *in vitro* model used here.[18] We show here that ipratropium causes massive destructuring of biofilms associated with a marked increase in activity of moxifloxacin and, to a lesser extent, solithromycin. Likewise, salbutamol also increases the activity of these two antibiotics, probably by stimulating bacterial neuraminidase activity, since most of its effects were antagonized by zanamivir, originally designed as an inhibitor of the influenza neuraminidase[26] but which also acts on pneumococcal neuraminidase A (NanA).[27]

Materials and methods

AECB patients and correlations between medication and severity factors

Forty-seven *S. pneumoniae* isolates were collected from patients with: (i) confirmed AECB diagnosis (Anthonisen's criteria);[28] (ii) a specimen fulfilling the interpretive criteria for lower respiratory tract origin;[29] (iii) need of hospitalization; and (iv) anamnestic confirmation of recent or current bronchodilator use. Data were thereafter anonymized. Tables S1 and S2 (available as Supplementary data at *JAC* Online) show the patients' main demographic, environmental and medical characteristics and the associations between pre-hospitalization medications and markers of disease severity. In brief, patients were almost equally distributed within the age groups of 55–64, 65–74 and ≥75 years. Comorbidities [diabetes, lung cancer (primary or with metastases), alcoholism, psychiatric disorders, hypertension] were frequent. Most patients were men and lived at home prior to hospitalization, and ∼60% were active smokers. Incidences of obstruction severity according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) scores[16] were almost equally distributed between low (1 or 2) and high (3 or 4) levels. Use of β_2-agonists, muscarinic antagonists, long-acting bronchodilators and inhaled corticoids prior to hospitalization were significantly associated with higher COPD severity (GOLD score 3 or 4) upon admission and prolonged hospitalization (>10 days). Short-acting bronchodilator intake was associated with high obstruction severity. The protocol for sample collection and subsequent analysis, and for access to the corresponding medical files, was approved by the *ad hoc* committees of our university and of the contributing clinical centres (unique Belgian number 40320109783).

Strains: origin, culture and antibiotic susceptibility testing

S. pneumoniae reference strains ATCC 49619 (capsulated, serotype 19F; used as international reference for pneumococcal susceptibility testing[30]) and R6 (non-capsulated; often used for *in vitro* studies of pneumococcal biofilm architecture[31] and of the implication of NanA in biofilm formation[32]) were purchased from ATCC (Manassas, VA, USA). The clinical isolate N6 was from the collection assembled for this study and originated from a typical COPD patient with respiratory tract colonization with both *S. pneumoniae* and *Haemophilus influenzae*, severe respiratory obstruction (GOLD score 3), two common comorbidities (hypertension and psychiatric disorders[33–35]), deep tobacco addiction and receiving Combivent® [a combination of fenoterol (a short-acting β_2-agonist with properties similar to those of salbutamol) and ipratropium] as pre-admission treatment. All strains were grown on agar plates and MICs were determined by microdilution following the recommendations of the CLSI.[30]

Biofilm models

Naive and induced biofilms were obtained exactly as previously described.[18] In brief: (i) 96-well plates were used as support and inoculated with $\sim 5 \times 10^7$ cfu/mL in cation-adjusted Mueller–Hinton broth supplemented with 5% lysed horse blood and 2% glucose; and (ii) naive biofilms were obtained by incubation for 2–11 days and induced biofilms by starting with an inoculum of the supernatant (free bacteria) from a 6-day-old biofilm. Biofilms were cultivated in control medium or in medium supplemented with ipratropium (1.45 mg/L), salbutamol (7.25 mg/L), zanamivir (250 mg/L) or their combination. These concentrations were chosen for the following reasons: (i) for ipratropium or salbutamol, to mimic those expected in the epithelium lining fluid of patients upon single administration of these drugs by inhalation; and (ii) for zanamivir, to obtain maximal inhibition of NanA (see Text S1).

Biomass and bacterial viability quantifications

Bacterial viability was assessed by the reduction of resazurin to fluorescent resorufin and biomass was quantified by crystal violet staining as previously described.[18]

Antibiotic effect on biomass and viability (pharmacodynamic model)

After 2 or 11 days of biofilm growth, culture media were removed and replaced with either fresh medium (negative control) or 1% SDS [to achieve complete bacterial killing and biomass solubilization (positive control)], or a medium supplemented with the antibiotic under study at concentrations ranging from 10^{-4} to 10^3 times its MIC in broth. After 24 h, residual biomass and bacterial viability were quantified and expressed as percentages of a negative control (no antibiotic added).[18] Data were used to fit a Hill equation (sigmoid) as a function of the antibiotic concentration to determine: (i) relative maximal efficacy [E_{max} (decrease in viability or biomass as a percentage of the control as extrapolated for an infinitely large antibiotic concentration)]; and (ii) relative potency [C_{50} (concentration as a multiple of the MIC in broth yielding a 50% reduction of the signal measured in the absence of antibiotic)], two key pharmacological descriptors of the activity of antibiotics against biofilms.[18]

Free sialic acid assay in biofilm supernatant

Free sialic acid was extracted from the supernatant and its concentration determined by both HPLC–MS and enzymatic[36] assay [linear correlation ($R^2 = 0.966$); slope (enzymatic/HPLC–MS) $= 0.841 \pm 0.059$; for details see Text S2].

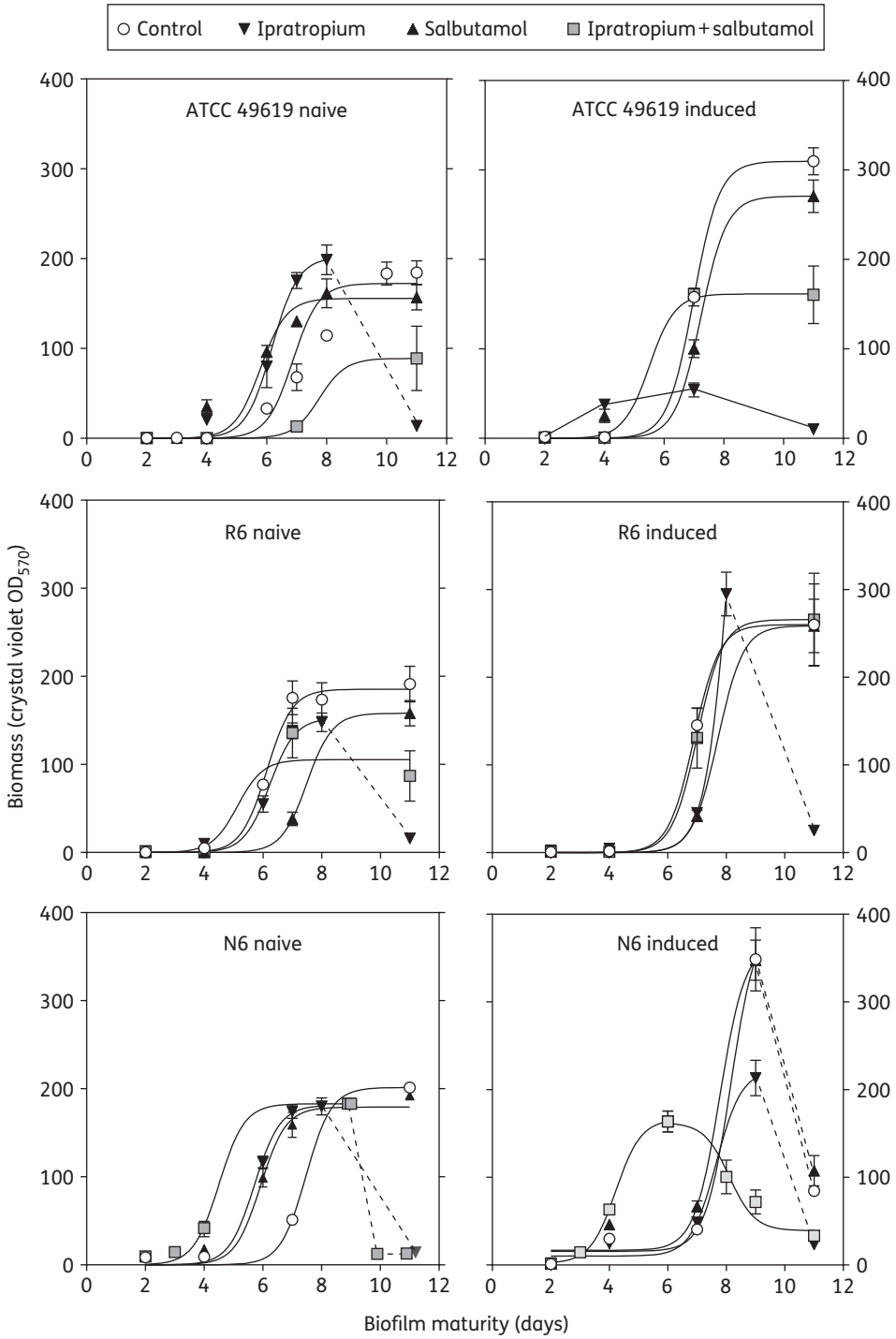

Figure 1. Kinetics of biofilm formation (biomass, as evaluated by crystal violet OD_{570}) by the reference capsulated strain ATCC 49619 (upper panels), the reference non-capsulated strain R6 (middle panels) and the clinical isolate N6 (lower panels), in the naive (left panels) and induced (right panels) models when cultured in control conditions (circles) or medium supplemented with 1.45 mg/L ipratropium (inverted triangles), 7.25 mg/L salbutamol (triangles) or the combination of ipratropium (1.45 mg/L) and salbutamol (7.25 mg/L) (squares). All values are means ± SEM of 3–26 experiments (each performed 12 times; when not visible, the SEM bars are smaller than the symbols). Data were used to fit a sigmoidal dose–response function whenever possible (broken straight lines are used when changes in OD_{570} occurred abruptly). An inverted sigmoidal function (slope factor = 1) was used to describe the decrease in OD_{570} observed between days 6 and 11 for the biofilm grown from strain N6 in the presence of the combination of ipratropium and salbutamol.

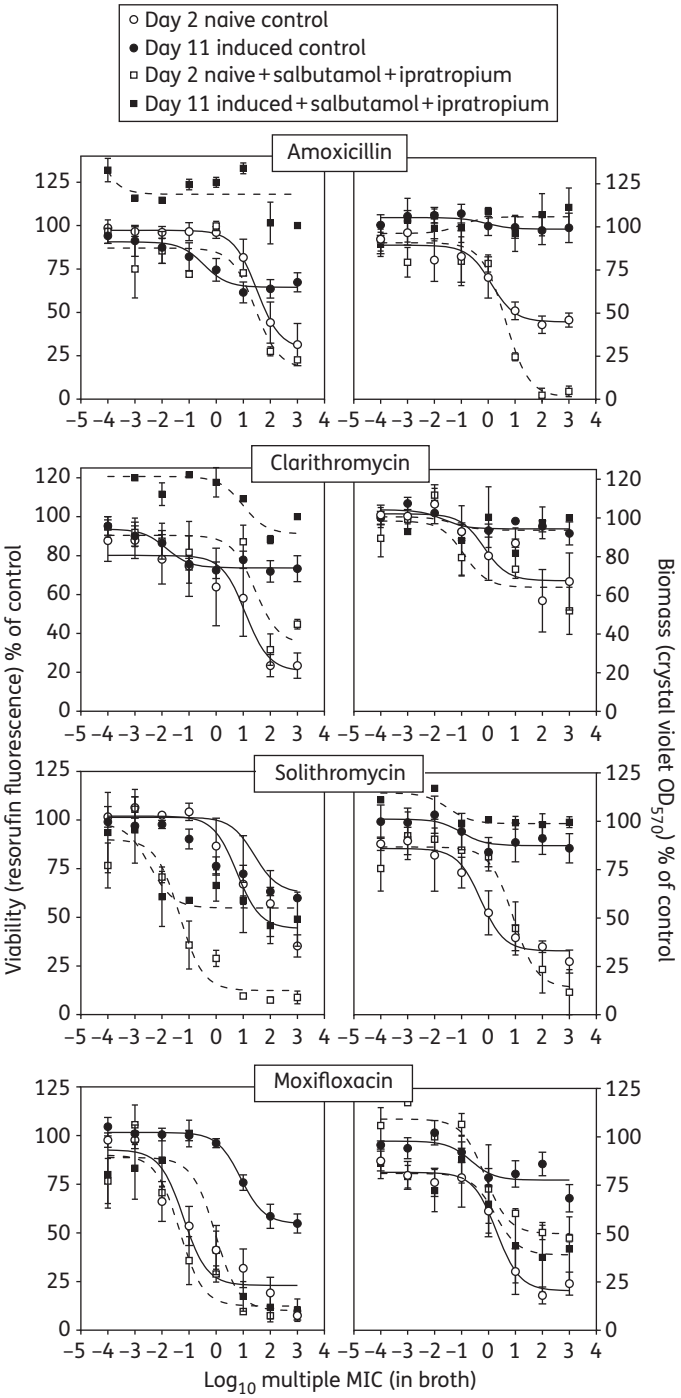

Figure 2. Concentration–response effects of amoxicillin, clarithromycin, solithromycin and moxifloxacin (from top to bottom) on viability (left) and biomass (right) of 2-day-old naive (open symbols) and 11-day-old induced (filled symbols) biofilms produced from strain ATCC 49619 grown in control conditions (circles and continuous lines) or in medium supplemented with the combination of ipratropium (1.45 mg/L) and salbutamol (7.25 mg/L) (squares and broken lines). The ordinate shows the change in viability (resorufin fluorescence) or biomass (crystal violet OD_{570}) as a percentage of the values observed in the absence of antibiotic. The abscissa shows the antibiotic concentration range investigated as multiples of the MICs (mg/L) of the corresponding drugs in broth (amoxicillin, 0.03; clarithromycin, 0.03; moxifloxacin, 0.125; solithromycin, 0.008). All values are means ± SEM of two to eight determinations, each performed in quadruplicate (when not visible, the SEM bars are smaller than the symbols). Data were used to fit sigmoidal dose–response curves (slope factor = 1; numerical values for the pertinent pharmacological descriptors E_{max} and C_{50} observed for biofilms exposed to moxifloxacin or solithromycin and a statistical analysis of their differences are presented in Tables S3, S4 and S5 for strains ATCC 49619, R6 and N6, respectively).

Release of sialic acid from S. pneumoniae by bacterial neuraminidase

Bacteria were incubated (3 h, 37°C) in PBS, pH 7.4, with or without purified *Arthrobacter ureafaciens* α-(2→3,6,8,9)-neuraminidase (Sigma-Aldrich, St Louis, MO, USA) in the presence or absence of salbutamol, zanamivir or their combination. The released sialic acid was then quantified by the enzymatic assay described above.

Pharmacological agents

Ipratropium and salbutamol were obtained as the solutions used for nebulization in standard patient care and distributed for clinical use in Belgium (Atrovent®, Boehringer Ingelheim, Ingelheim am Rhein, Germany; and Ventolin®, GlaxoSmithKline, Genval, Belgium, respectively) and complying with the provisions of the European Pharmacopoeia. These solutions were diluted with culture medium to reach the appropriate concentrations needed for our experiments. Zanamivir was purchased from Sigma-Aldrich. Amoxicillin was obtained as the branded product for human parenteral use complying with the provisions of the European Pharmacopoeia (>90% purity) and distributed in Belgium as Clamoxyl® by GlaxoSmithKline s.a./n.v. (Genval, Belgium). Clarithromycin, moxifloxacin and solithromycin were obtained as microbiological standards (purity 100%) from Teva Pharmaceutical Industries (Petah Tikva, Israel), Bayer Schering Pharma AG (Berlin, Germany) and Cempra Pharmaceuticals (Chapel Hill, NC, USA), respectively.

Curve fitting, correlations and statistical analyses

These were performed with GraphPad Prism® 4.03 and 6.05 and GraphPad Instat® 3.10 (GraphPad software, San Diego, CA, USA) or JMP® 10.0.2 (SAS Institute Inc., Cary, NC, USA). A P value <0.05 was considered to indicate a significant difference between groups or datasets.

Results

Biofilm production by clinical isolates: relation to COPD severity and patient medications

Naive biofilms were generated from all clinical strains and biomass was quantified after 10 days of culture in control medium. Results were stratified according to: (i) COPD severity upon admission (using GOLD scores[16]); and (ii) patients' bronchodilator medication prior to hospitalization. No significant association between biomass amounts and GOLD scores was found but strains collected from patients who had received only muscarinic antagonists produced significantly less biomass than strains from patients who had received no treatment or other bronchodilator(s) (one-way ANOVA with Tukey's post test, $P<0.05$).

Influence of bronchodilators on biofilm formation from selected strains

Because no correlation between the rate of biofilm formation and the severity of patients' respiratory obstruction could be observed

amongst the clinical isolates, we selected one of them [N6 (capsulated; serotype 35B); see the Materials and methods section for a description of the patient] for all subsequent experiments.

As previously shown,[18] biomass reached larger values over time in the induced than in the naive model for all three strains (Figure 1). Of note, for the clinical strain, the intense growth

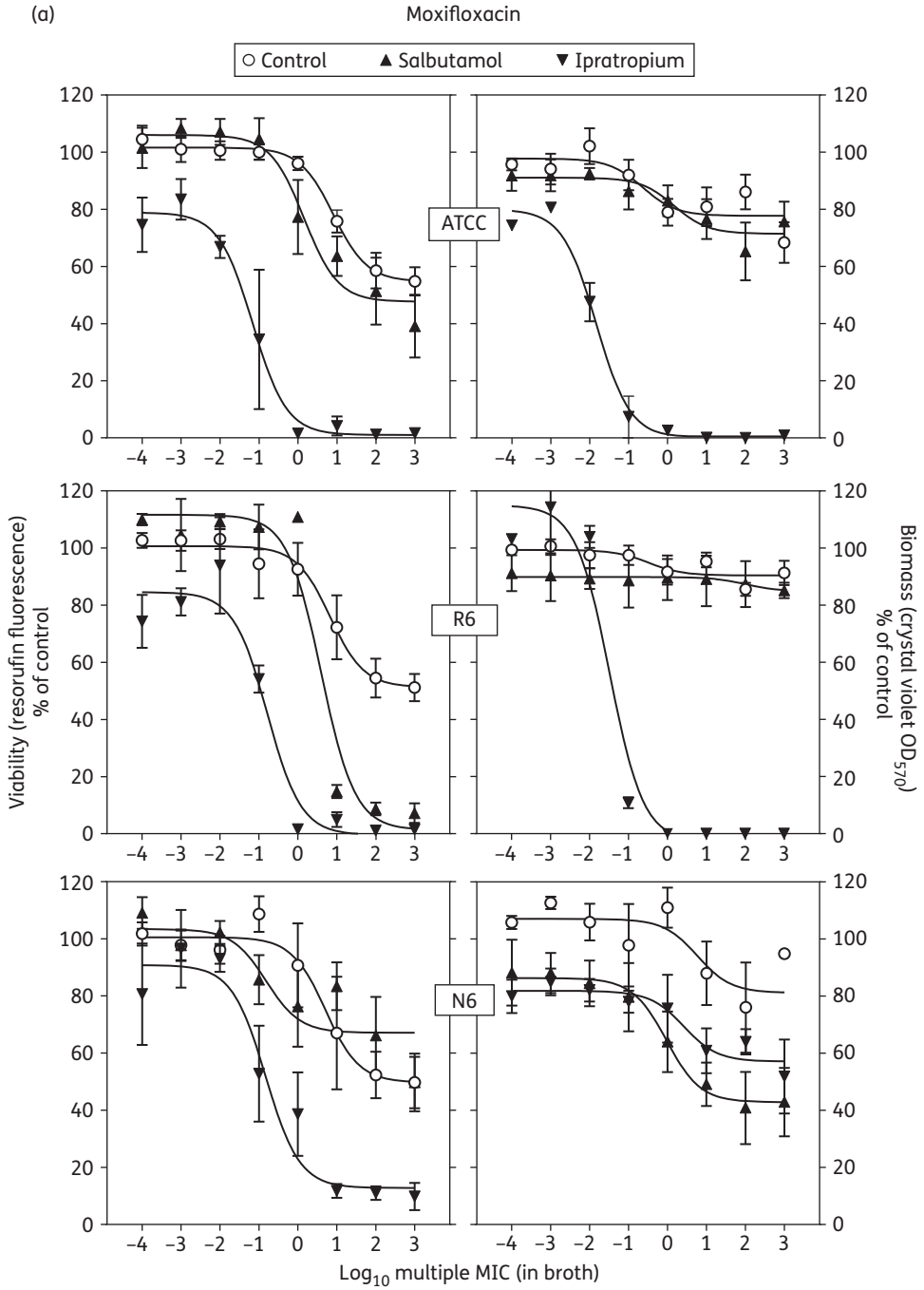

Figure 3. Concentration–response effects of moxifloxacin (a) and solithromycin (b) on viability (left) and biomass (right) of 11-day-old induced biofilms produced from strain ATCC 49619, R6 or N6 (from top to bottom) grown in control conditions (circles) or in the presence of 7.25 mg/L salbutamol (triangles) or 1.45 mg/L ipratropium (inverted triangles). The ordinate shows the change in viability (resorufin fluorescence) or biomass (crystal violet OD_{570}) as a percentage of the value observed in the absence of antibiotic. The abscissa shows the concentration range investigated as multiples of the MIC of the corresponding antibiotics in broth [0.125 and 0.008 mg/L (ATCC 49619), 0.064 and 0.004 mg/L (R6) and 0.064 and 0.004 mg/L (N6) for moxifloxacin and solithromycin, respectively]. All values are means ± SEM of two to eight determinations, each performed in quadruplicate (when not visible, the SEM bars are smaller than the symbols). Data were used to fit sigmoidal dose–response curves (slope factor = 1; numerical values for the pertinent pharmacological descriptors E_{max} and C_{50} and a statistical analysis of their differences are presented in Tables S3, S4 and S5 for strains ATCC 49619, R6 and N6, respectively).

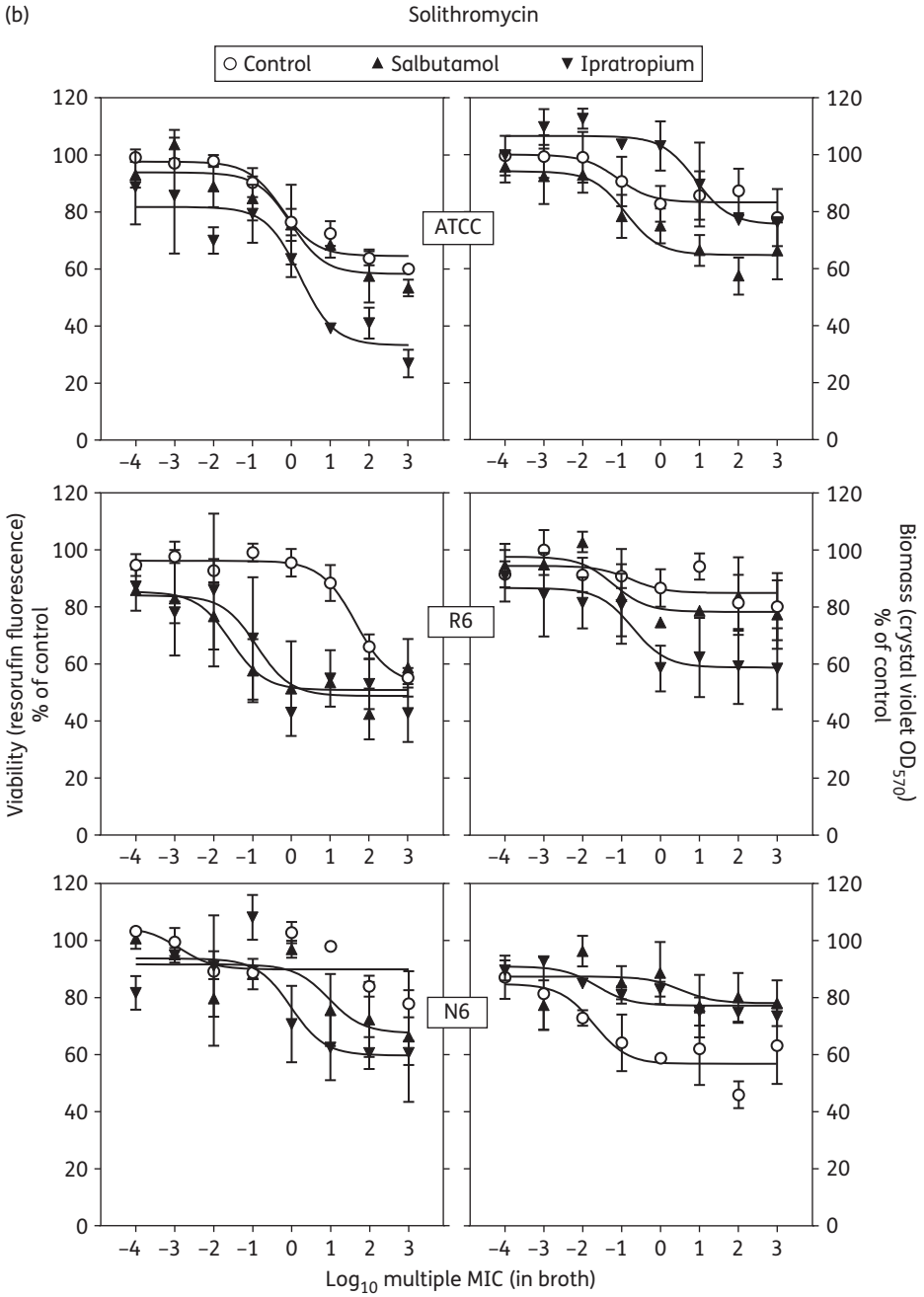

Figure 3. *Continued*

obtained in the induced model at day 9 was followed by a precipitous loss of biomass at day 11, consistent with the well-known disassembly process leading to dissemination of the bacteria.[37] For the naive model, addition of ipratropium did not affect biomass increase up to day 8 but was subsequently associated with an almost complete loss of biomass at day 11 for all three strains. In the induced model, ipratropium: (i) also caused a precipitous loss of biomass at day 11 for the reference R6 strain; (ii) induced marked inhibition of biomass formation for the reference strain ATCC 49619 at day 7 and a loss of biomass subsequently; and (iii) impaired the formation of biomass at day 9 and caused an almost complete loss of this biomass at day 11 for the clinical N6 strain. In sharp contrast, salbutamol did not have a marked effect on biomass over time compared with the control. The combination of ipratropium and salbutamol: (i) reduced the amount of biomass at day 11 for naive and induced biofilms obtained from strain ATCC 49619 and for naive biofilms obtained from strain R6; and (ii) caused a loss of biomass at day 10 for the naive biofilms and from day 8 for induced biofilms obtained from the clinical strain N6.

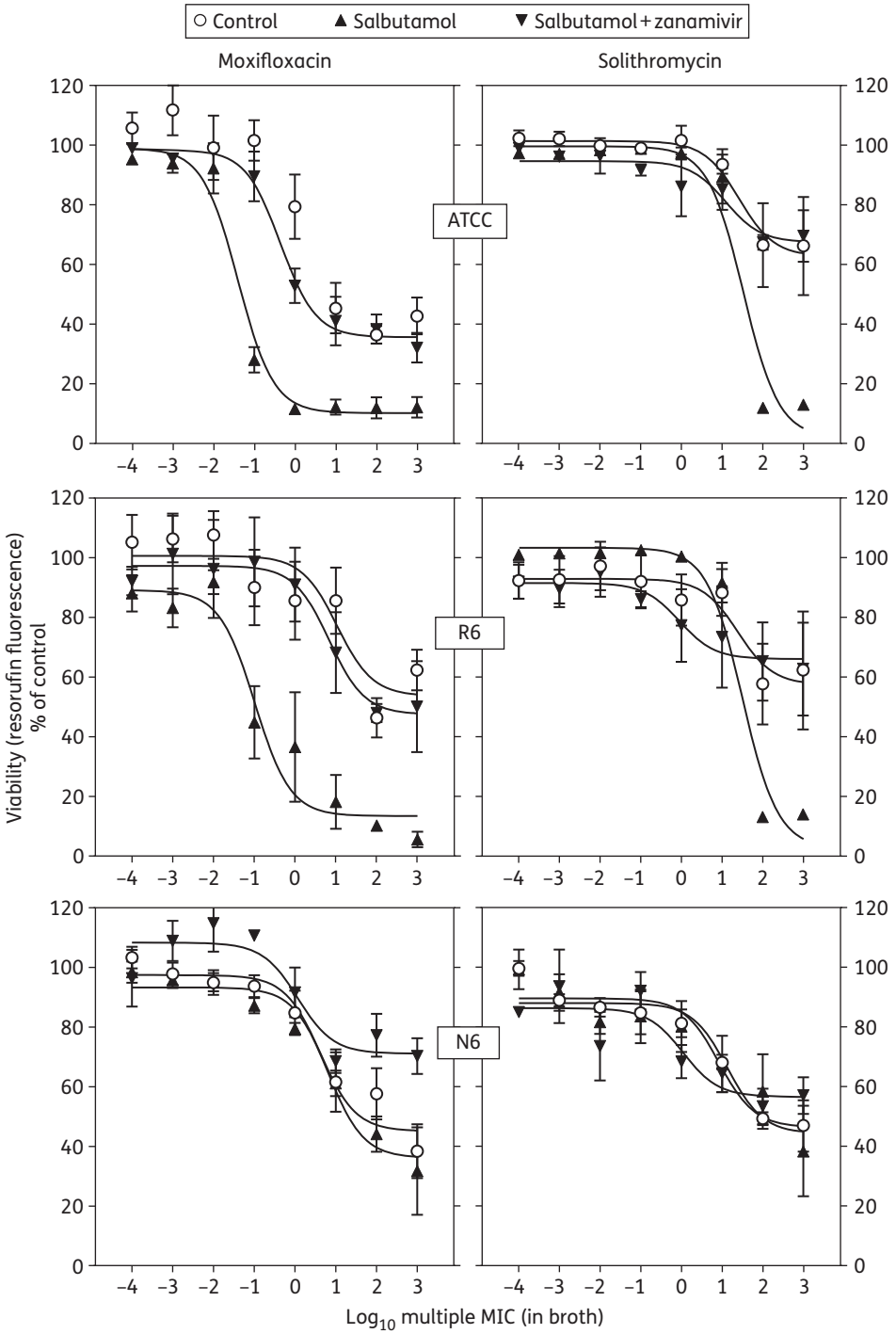

Figure 4. Concentration–response effects of moxifloxacin (left panels) and solithromycin (right panels) on viability against 2-day-old induced biofilms produced from strain ATCC 49619, R6 or N6 (from top to bottom) grown in control conditions (circles) or in the presence of 7.25 mg/L salbutamol (triangles) or 7.25 mg/L salbutamol + 250 mg/L zanamivir (inverted triangles). The ordinate shows the change in viability (resorufin fluorescence) as a percentage of the value observed in the absence of antibiotic. The abscissa shows the concentration range investigated as multiples of the MICs of the corresponding antibiotics in broth [0.125 and 0.008 mg/L (ATCC 49619), 0.064 and 0.004 mg/L (R6) and 0.064 and 0.004 mg/L (N6) for moxifloxacin and solithromycin, respectively]. All values are means ± SEM of two to eight determinations, each performed in quadruplicate (when not visible, the SEM bars are smaller than the symbols). Data were used to fit sigmoidal dose–response curves (slope factor = 1; numerical values for the pertinent pharmacological descriptors E_{max} and C_{50} and a statistical analysis of their differences are presented in Tables S3, S4 and S5 for strains ATCC 49619, R6, and N6, respectively).

Activity of amoxicillin, clarithromycin, moxifloxacin and solithromycin against biofilms grown in control conditions or in the presence of salbutamol combined with ipratropium

We first examined whether growing biofilms in the presence of the combination of salbutamol and ipratropium modified the activity of antibiotics with respect to both bacterial viability and biomass using the ATCC 49619 reference strain and both 2 day naive and 11 day induced biofilms (Figure 2). For amoxicillin and clarithromycin, only modest effects were observed with 2 day naive biofilms and no effect with 11 day induced biofilms. Conversely, moxifloxacin and solithromycin activities were markedly enhanced with respect to both E_{max} and C_{50}, especially if considering bacterial viability (for numerical data see Tables S3–S5). To check for absence of direct antibiotic effects of ipratropium or of salbutamol on *S. pneumoniae*, we measured the MICs of moxifloxacin and solithromycin in the presence of these bronchodilators at concentrations up to 4 and 8 mg/L, respectively, and saw no effect. Based on this first set of observations, only moxifloxacin and solithromycin were used in further studies.

Analysis of the changes in moxifloxacin and solithromycin activity against biofilms grown in the presence of ipratropium (alone) or salbutamol (alone or with zanamivir)

In the 11 day biofilm model (Figure 3a), moxifloxacin completely suppressed the viability signal (reduction of resazurin) when biofilms developing from strain ATCC 49619, R6 or N6 had been grown in the presence of ipratropium alone compared with a maximal effect of only ~50% in controls. This, interestingly enough, was even better than the result observed for the highly susceptible 2 day naive biofilms in non-supplemented medium (control; compare with Figure 2). Similar effects on biomass were observed for the reference ATCC 49619 and R6 strains, but to a much lesser extent for the clinical strain N6. Moreover, the C_{50} of moxifloxacin was also improved by ipratropium. For solithromycin (Figure 3b), growing biofilms in the presence of ipratropium exerted an effect that was qualitatively similar to that observed for moxifloxacin but quantitatively less marked.

In contrast, growing biofilms in the presence of salbutamol alone was without marked effect on moxifloxacin or solithromycin activity against the 11 day induced biofilm, except for viability with the reference R6 strain (Figure 3a and b). However, when tested in the 2 day induced model, salbutamol improved moxifloxacin and solithromycin killing activities against biofilms formed by the two reference strains, and this effect was blocked by zanamivir (Figure 4). We checked that zanamivir (up to a concentration of 8 mg/L) did not modify the MIC for bacteria growing in broth. Tables S3–S5 and Figures S1–S6 show numerical data and graphical comparisons of the E_{max} and C_{50} values for each of the above conditions together with a statistical analysis of their differences.

Influence of biofilm pre-exposure to bronchodilators on sialic acid release in biofilm supernatant

Since biofilm cohesion depends on sialic acid-mediated intrabacterial bonds,[38] we checked whether the biomass decrease observed with biofilms grown in the presence of ipratropium

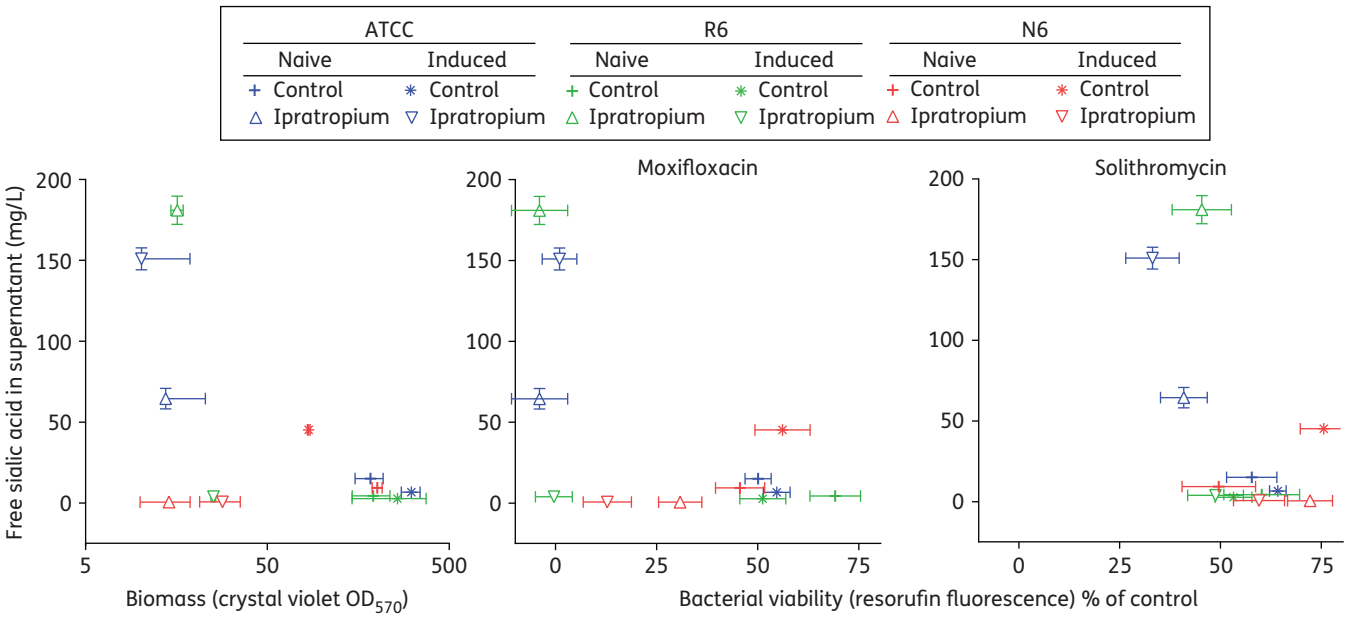

Figure 5. Correlations between free sialic acid concentrations in the supernatant of biofilms and (i) biomass and (ii) bacterial viability using data from Figure 3. Conditions: 11-day-old naive (crosses and triangles) or induced (asterisks and inverted triangles) biofilms grown in control medium (crosses and asterisks) or in medium supplemented with ipratropium (1.45 mg/L; triangles and inverted triangles) produced by strain ATCC 49619 (blue symbols), R6 (green symbols) or N6 (red symbols). Left panel: data are presented as a function of biomass. Middle and right panels: data are presented as a function of the bacterial residual viability after incubation with moxifloxacin (middle panel) or solithromycin (right panel). Each data point is the mean ± SEM of three to eight independent experiments, each performed in quadruplicate (horizontal bars in the left diagram that do not extend to both sides of the symbol correspond to a range that would go beyond the left boundary of the graph; note that the abscissa is logarithmic). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

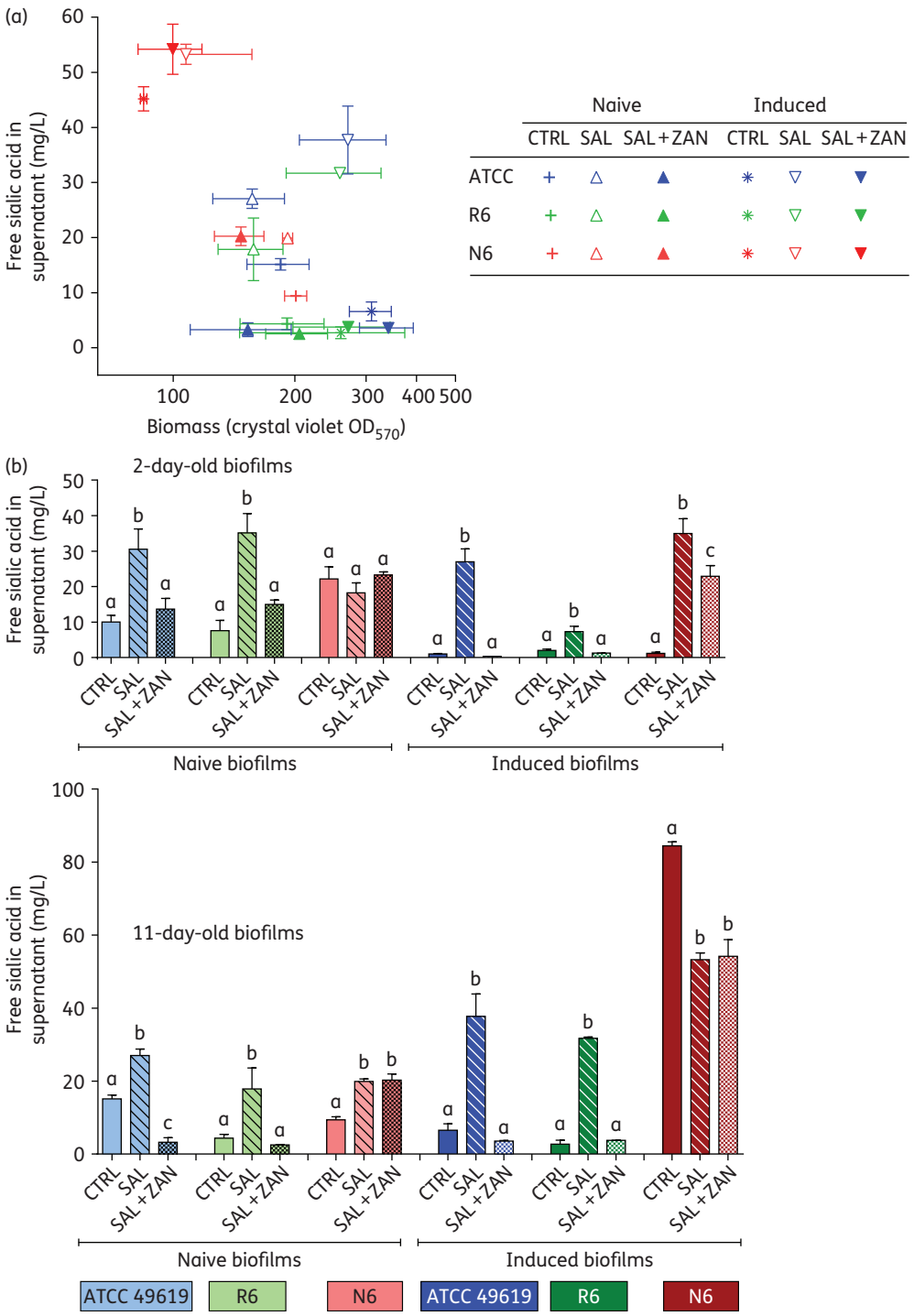

Figure 6. (a) Correlation between the concentration of free sialic acid in the supernatant and biomass. Conditions: 11-day-old naive (crosses and triangles) or induced (asterisks and inverted triangles) biofilms developing from strains ATCC 49619 (blue symbols), R6 (green symbols) or N6 (red symbols) grown in control medium (CTRL; crosses and asterisks) or medium supplemented with salbutamol (SAL; 7.25 mg/L; open triangles and open inverted triangles) or with the combination of salbutamol (7.25 mg/L) and zanamivir (250 mg/L) (SAL+ZAN; filled triangles and filled inverted triangles) and biomass amounts. (b) Influence of salbutamol (SAL; 7.25 mg/L) or salbutamol (7.25 mg/L) combined with zanamivir (250 mg/L) (SAL+ZAN) on the concentration of free sialic acid in the biofilm supernatant as determined for each strain individually and, for each of them, for 2 day (upper histogram) and 11 day (lower histogram) naive and induced biofilms. CTRL, control. Statistical analysis (one-way ANOVA with Tukey's post test): in each group, bars with different letters indicate significant differences between media ($P<0.05$). Each data point is the mean ± SEM of three to eight independent experiments, each performed in quadruplicate. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

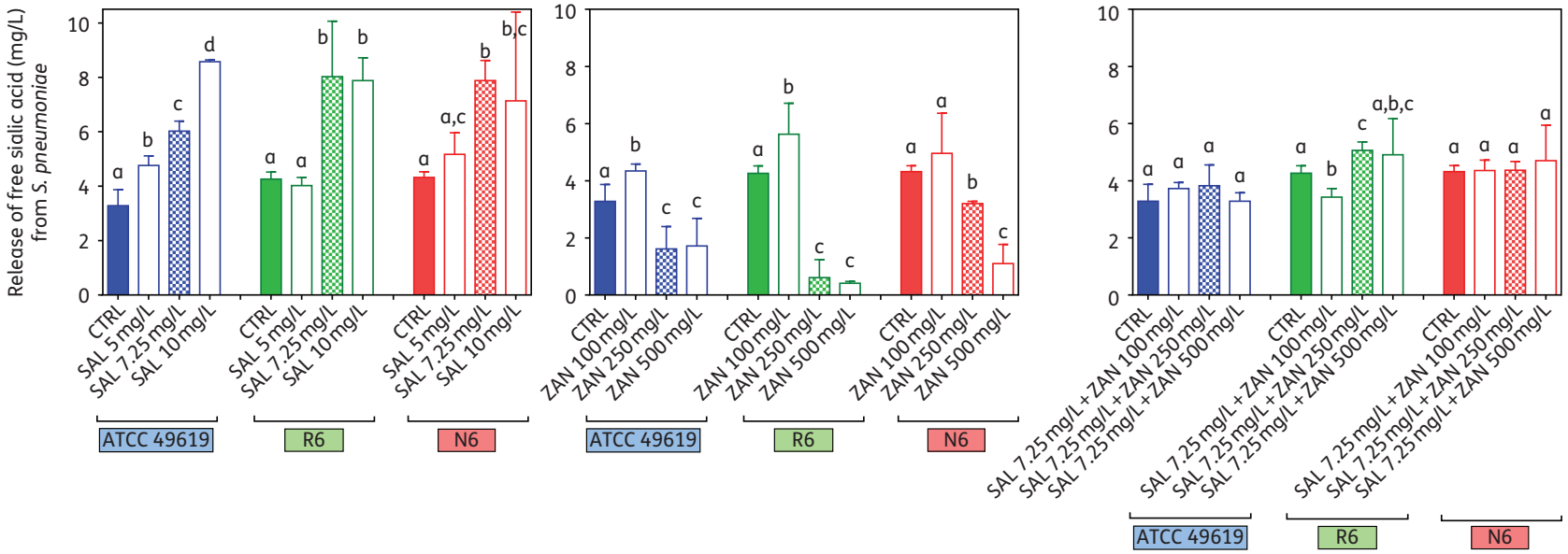

Figure 7. Amounts of sialic acid (mg/L) released from *S. pneumoniae* collected from the supernatant of 2-day-old naive biofilms [developing from strains ATCC 49619 (blue), R6 (green) or N6 (red)] by purified *A. ureafaciens* α-(2→3,6,8,9)-neuraminidase alone or in the presence of salbutamol, zanamivir or their combination. CTRL (full bars), control conditions (no addition); SAL, ZAN, SAL + ZAN, addition of salbutamol, zanamivir or their combination at the concentrations indicated on the abscissa. Statistical analysis (one-way ANOVA with Tukey's post test): in each group, bars with different letters indicate significant differences between conditions ($P < 0.05$). Salbutamol and/or zanamivir concentrations used for biofilm culture during studies of biofilm development and antibiotic activity are represented by stippled bars; other concentrations used in this experiment are represented by open bars. Data are means ± SD for one experiment performed in triplicate. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

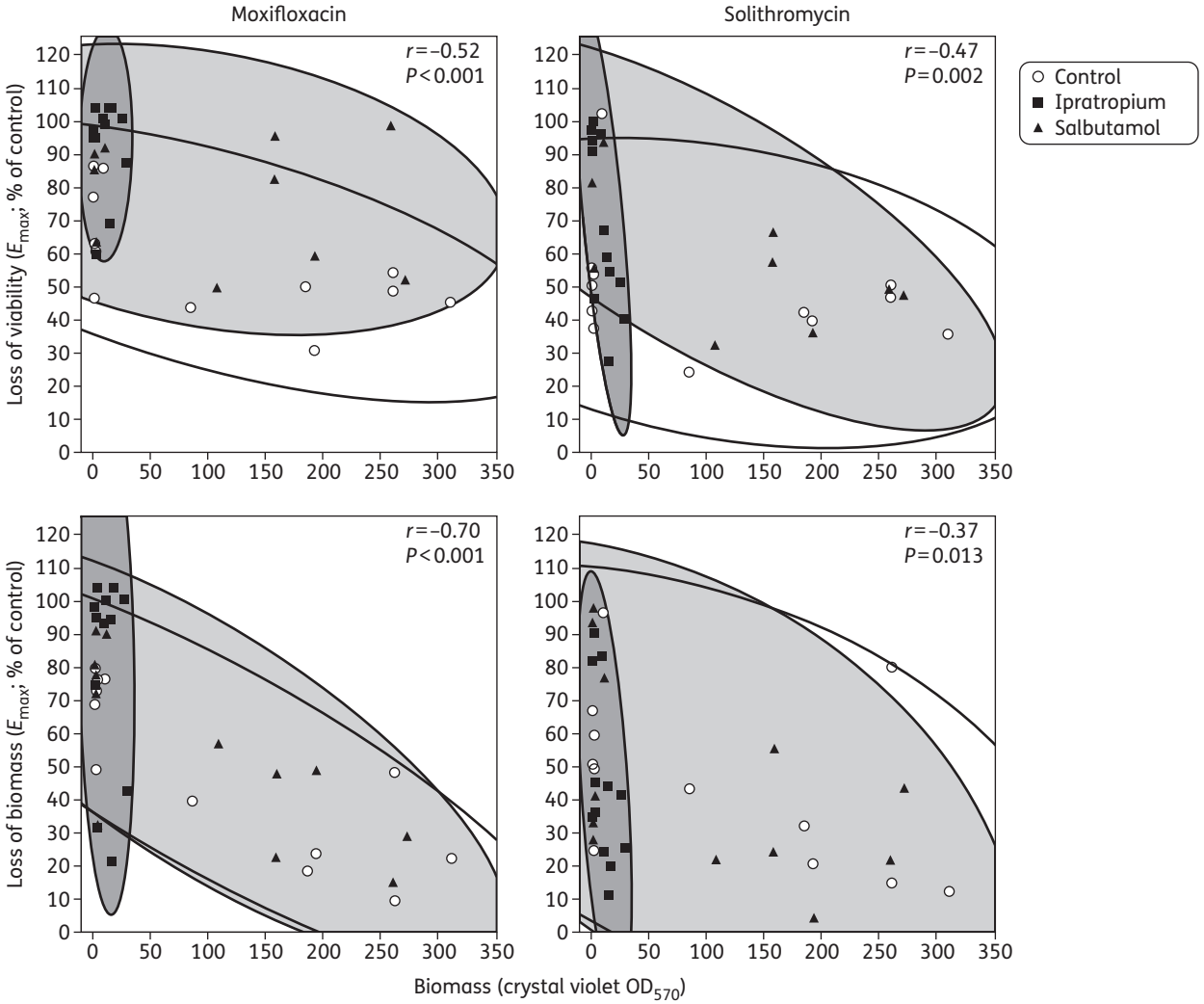

Figure 8. Correlation between maximal losses of viability (upper panels) and of biomass (lower panels) and biomass in 2 day and 11 day naive and induced biofilms formed from all three strains used in the study (reference strains ATCC 49619 and R6 and clinical strain N6) when grown in control medium (circles) or in the presence of 1.45 mg/L ipratropium (squares) or 7.25 mg/L salbutamol (triangles) and exposed to moxifloxacin (left panels) or solithromycin (right panel). The ordinate shows the antibiotic E_{max} observed for each condition in the pharmacodynamic model (see Figures 3, S1, S3 and S5 and Tables S3, S4 and S5). The abscissa shows the amount of biomass as assessed by crystal violet staining (OD_{570}). The ellipses show the 95% probability area for each correlation (white, control medium; dark grey, ipratropium; light grey, salbutamol). Pearson correlation coefficients (*r*) and the corresponding probability values {*P* values [one tail, because there was no evidence of an inverse (positive) correlation]} were calculated considering the whole dataset in each panel.

and their increased susceptibility to antibiotics were associated with the release of free sialic acid in the medium. Figure 5 (left panel) shows the amount of sialic acid released in the medium of 11-day-old biofilms as a function of biofilm biomass. Sialic acid was released in larger amounts when biofilms had been grown in the presence of ipratropium, which was accompanied by a reduction in biomass for the two reference strains (ATCC 49619 and R6) compared with control conditions. For the clinical strain, N6, little sialic acid release was detected at day 11 because of the complete destructuring of the biofilm that had already been achieved earlier (Figure 1) and had been accompanied by massive sialic acid release between days 2 and 7 (data not shown). Therefore, no association could be established between this release and biomass. Figure 5 (middle and right panels) shows that this release of sialic acid was associated with a reduction in the viability of biofilms exposed to moxifloxacin and, to a lesser extent, to solithromycin for the two reference strains but not for the N6 strain.

Since we had observed that zanamivir completely abolished the effect of salbutamol on antibiotic efficacy (Figure 4), we examined in more detail the changes in sialic acid release in biofilms exposed to salbutamol with and without zanamivir. Focusing first on 11 day biofilms (to compare with ipratropium), no clear correlation was seen between free sialic acid levels and biomass (Figure 6a), partly because of high variability between strains. These were, therefore, examined individually for both 2- and 11-day-old biofilms and for naive and induced biofilms. Figure 6(b) shows that exposure to salbutamol systematically increased sialic

acid release for biofilms formed by the reference strains (ATCC 49619 and R6) and that this increase was suppressed by zanamivir. For the clinical N6 strain, salbutamol only increased sialic acid release for the 2-day-old induced biofilm, and zanamivir exerted no or little effect.

Modulation of bacterial neuraminidase activity by salbutamol and zanamivir measured by sialic acid release from S. pneumoniae

Because the effects of salbutamol on sialic acid release and antibiotic activity were reversed by zanamivir, we examined whether salbutamol could directly modulate the activity of a purified neuraminidase against *S. pneumoniae*. Figure 7 shows the activity of neuraminidase against the three strains investigated in control conditions and in the presence of increasing concentrations of salbutamol, zanamivir and their combinations. Enzymatic activity was increased in the presence of salbutamol, with significant effects obtained at the concentration used in the biofilm experiments. Conversely, zanamivir reduced neuraminidase activity and also reversed the stimulating effect of salbutamol.

Discussion

To the best of our knowledge, this study demonstrates for the first time that culturing pneumococcal biofilms in the presence of two major and widely used bronchodilators markedly modulates their cohesion and their susceptibility to two antibiotics: moxifloxacin and solithromycin. In contrast, little effect was seen for amoxicillin and clarithromycin. The most striking results were observed with ipratropium, although those seen with salbutamol were not negligible. These are globally depicted in Figure 8.

Ipratropium and other choline analogues are known to interact with *S. pneumoniae* choline-binding proteins, including LytA amidase, LytC lysozyme and Pce phosphocholinesterase.[39] LytA and LytC play a critical role in pneumococcal attachment to epithelia, tissue colonization and biofilm formation.[31,40] We show here that ipratropium exerts major effects on the matrix of aged biofilms accompanied or even preceded by a massive release of sialic acid, consistent with a process of biofilm disassembly. Our data strongly suggest that this disassembly contributes to the increased activity of moxifloxacin and, to a lesser extent, of solithromycin, probably by improving access of the antibiotics to bacteria. The lesser and even negligible effects of amoxicillin and clarithromycin that were observed during the first stages of our study could be related to the intrinsically poor activity of these antibiotics against *S. pneumoniae* biofilms.[18]

Moving now to salbutamol, the data suggest that it mainly acts through matrix remodelling mediated by the activation of neuraminidase, which may facilitate antibiotic diffusion. Indeed, zanamivir, known to inhibit pneumococcal NanA,[27] abolishes the release of sialic acid induced by salbutamol and its enhancing effect on antibiotic activity. Both the stimulatory effect of salbutamol and the inhibitory effect of zanamivir could be reproduced *in vitro* with purified neuraminidase. In *S. pneumoniae*, NanA contributes to biofilm formation by cleaving sialic acid residues from glycans and mucin at the epithelial cell surface, thus exposing host cell surface receptors for pneumococcal adherence.[32,41–43] Sialic acid itself can also act as a signalling molecule, enhancing bacterial adherence to surfaces and/or survival within biofilms.[38] Moreover, sialic acid is present in the intercellular matrix of pneumococcal biofilms.[44] As sialylated moieties are present on or between bacteria,[45] the presence of free sialic acid in biofilm supernatants suggests a remodelling of the three-dimensional structure of the matrix and/or weakening of the interactions between bacteria during maturation.

Our study had three main limitations: (i) the small number of strains examined; (ii) the use of a single molecule as representative of each bronchodilator and antibiotic class; and (iii) the artificial nature of the support used for growing biofilms. Moreover, the effects of both ipratropium and salbutamol on antibiotic activity were less marked for biofilms formed from the clinical isolate, N6, compared with those formed from the two reference strains. This may have resulted from differences in matrix composition or three-dimensional structure, which are strain- and serotype-dependent.[5,44,46] Moreover, the effects of salbutamol may also depend on the level of activity of NanA, which varies among serotypes, as described for other streptococcal species.[47] With respect to testing for additional bronchodilators, we unfortunately could not examine long-acting β_2-agonists (e.g. salmeterol) or selective long-acting M2–M3 muscarinic antagonists (e.g. tiotropium) because these compounds are sparingly water soluble (for details see Drug Bank, http://www.drugbank.ca/) and are unavailable as commercial solutions, making it difficult to use them in our *in vitro* model. In addition, we could not test for an effect of corticosteroids because we observed that budesonide, a typical inhaled corticosteroid, itself has an antibacterial effect on planktonic cells (with MICs of ~3–6 mg/L), confirming literature data.[48] Our results as they are, however, clearly demonstrate the beneficial effect of ipratropium and, to some extent, salbutamol and their synergy with moxifloxacin and solithromycin. The observation that the two bronchodilators are not antagonists is of interest in this context. The marked effects seen with moxifloxacin may be related to the well-known intense bactericidal activity of this drug against *S. pneumoniae*.[49] It is tempting to speculate that the effects of moxifloxacin described here may at least partly explain why this antibiotic showed superiority to amoxicillin or clarithromycin for clinical cure, bacteriological eradication and long-term outcomes of AECBs in patients with COPD.[21] Similar clinical studies with solithromycin, which binds more tightly to the 50S ribosome subunit than clarithromycin,[50] would therefore be of great interest.

In conclusion, we show here for the first time that ipratropium and salbutamol, which are used as first-line therapy in most COPD patients with AECBs, may act not only *in vivo* through their primary and well-known action of decreasing bronchial smooth constriction, but also by contributing to the elimination of the pneumococcal biofilm, as demonstrated here *in vitro*. These drugs may also improve the activity of at least two antibiotics of distinct pharmacological classes in killing bacteria encased in biofilms and reducing biofilm mass. Globally, the present data may help to further support and rationalize the current GOLD guidelines, which recommend combining antibiotics with short-acting bronchodilators for the treatment of bacterial exacerbations[16] in grade A patients (as first choice) or for all grades as alternative treatments. They may also pave the way for more rational searching and screening for add-on therapies for AECBs, which, due to their recurrent character and the damage they cause to the bronchial tree, are largely responsible for the progressive and irreversible decline in respiratory function of affected patients.

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Transparency declarations

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Supplementary data

Text S1, Text S2, Tables S1 to S5 and Figures S1 to S6 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Modulation of the activity of moxifloxacin and solithromycin in an *in vitro* pharmacodynamic model of *S. pneumonia* naive and induced biofilms

Nathalie M. Vandevelde[1], Paul M. Tulkens[1], Giulio G. Muccioli[2], Françoise Van Bambeke[1,*]

A. Texts

Text S1: Choice of salbutamol, ipratropium and zanamivir concentrations used in the study

The concentrations of salbutamol (7.25mg/L) and ipratropium (1.45 mg/L) were chosen to mimic those expected in the epithelium lining fluid of patients after single administration of the drugs by inhalation (single puff; salbutamol: 2.5mg]; ipratropium: 0.5mg) considering (i) a mean pulmonary deposition of 10% (see Summary of Product Characteristics of Combivent® [association salbutamol+ipratropium],[1] and (ii) a mean epithelial lining fluid volume of 34.5 mL.[2] Zanamivir, an inhibitor of pneumococcal neuraminidase, was used at a concentration of 250 mg/L after pilot studies that it provided a maximal inhibition, as reported in the literature.[3]

Text S2: Assay of free sialic acid in biofilm supernatant (enzymatic and high performance liquid chromatography/mass spectrometry [HPLC-MS] assays

Biofilm supernatant was centrifuged at 14,000 RPM for 10 min (Eppendorf centrifuge 5417R, rotor DL 039, Eppendorf AG, Hamburg, Germany) and the resulting supernatant mixed with an equal volume of dimethylcetone (acetone; 98.5% purity; Merck AG, Darmstadt, Germany]). After centrifugation again at 14,000 RPM for 10 min, the resulting supernatant was collected, flushed with a gentle flow of air at room temperature until removal of the dimethylcetone. The residual aqueous phase was again mixed with an equal volume of dimethycetone and subjected to the same process 3 times. The final aqueous phase used assay as follows:

- Enzymatic assay: use of the Sialic Acid Quantification kit (Sigma-Aldrich, St Louis, MO) as instructed by its provider.[4]
- HPLC-MS assay: LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, Waltham, MA) coupled to an Accela HPLC system (ThermoFisher Scientific, Waltham, MA). HPLC specifications: stationary phase, Luna-NH2 (5µm) (150x2mm) column (Phenomenex Inc, Torrance, CA); mobile phases, acetonitrile containing 0.1% formic acid (A) and 5mM ammonium acetate containing 0.1% acetic acid (B); flow: 0.4 mL/min with (i) a gradient from 10% B to 70% B linearly over 15 min, (ii) 70% B over 15 min, (iii) re-equilibration at 10% B for 10 min. MS analysis was performed in the negative mode with an electrospray ionization (ESI) source. Blank samples were injected between each analysis to avoid carry over effects. Sialic acid levels were normalized *vs*. the signal obtained with the internal standard (zanamivir 0.1% m/v; used to check for accuracy of the method and then added to selected samples before purification).

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B. Tables

Table S1: Patients' demographic, environmental and medical characteristics (n=47)

age and no. enrolled				
mean	**<55 y**	**≥55 to <65**	**≥65 to <75**	**≥75 y**
68.7 ± 11.7	6 (13%)	13 (27.5%)	13 (27.5%)	15 (32%)
comorbidities				
cancer [b] %	**diabetes [c] %**	**alcoholism [d] %**	**Psychiatric disorders [e] %**	**hypertension [f] %**
19	21	30	36	62
general information and GOLD score				
gender % (M / F)	**living place % (home / nursing home / psychiatric institution)**	**smoking habits % [a] (active / former / non smoker / unknown)**		**GOLD score % (1-2 / 3-4)**
74 / 26	87 / 4.5 / 8.5	57 / 30 / 6.5 / 6.5		45 / 55

[a] according to patient's declaration

[b] tissue biopsies and/or chest x-rays

[c] fasting glycaemia > 1.26g/L

[d] according to patient's declaration, evidence at admission (inebriated condition), or presence of alcoholic cirrhosis

[e] Medical diagnosis of anxiety, depression or schizophrenia

[f] systolic blood pressure > 120mm Hg

Table S2: Associations between patients' medications and markers of severity.

Variables #1 relate to patients' most frequent medications and variables #2 to all other pertinent variables recorded in the study. Associations were tested by means of 2×2 contingency tables to calculate odd ratios (ORs) with the corresponding 95% confidence interval (CI) and p-value (Fisher's exact two-tailed test). The table shows only associations for which the p-value was <0.05. The number of patients with the corresponding variables is shown between brackets (total no. = 47).

Patients medication (variable #1)	**Odds ratios (95% IC) and *p*-value (variables #2)**	
	GOLD score 3-4 (n=26)	**Hospitalization > 10 days** (n=16)
β_2-agonist(s) [a] (n=33)	4.768 (1.887-12.046) p<0.001	0.245 (0.099-0.607) p<0.01
Muscarinic antagonist(s) [b] (n=33)	3.109 (1.278-7.566) p<0.05	0.373 (0.153-0.906) p<0.05
Short-acting bronchodilator [c] (n=23)	3.238 (1.419-7.388) p<0.01	1.520 (0.661-3.495) ns
Long-acting bronchodilator [d] (n=32)	3. 735 (1.540-9.059) p<0.01	0.194 (0.078-0.480) p<0.001
Inhaled Corticoids [e] (n=33)	3. 735 (1.540-9.059) p<0.01	0.294 (0.121-0.713) p<0.01
N-acetylcysteine (n=15)	2.875 (1.162-7.115) p<0.05	0.761 (0.293-1.978) ns

[a] salbutamol, fenoterol, formoterol, salmeterol, indacaterol

[b] ipratropium, thiotropium

[c] salbutamol, fenoterol [withdrawn in 2012], ipratropium

[d] formoterol, salmeterol, indacaterol, thiotropium

[e] budesonide, fluticasone, beclomethasone

Table S3. Pertinent regression parameters[a] with 95% confidence intervals and statistical analysis[c] for strain ATCC49619

Media	Biofilm models	Antibiotics	Effect on viability within the matrix			Effect on biofilm thickness		
			E_{max}[b] % loss of viability (CI at 95%)	Concentration (X MIC - mg/L) yielding 50% reduction	R^2	E_{max}[b] % loss of matrix (CI at 95%)	Concentration (X MIC- mg/L) yielding 50% reduction	R^2
CTRL	2 days naïve	MXF	77.0 (67.7 - 86.3) / (A; a)	0.10 (0.02 – 0.58) /0.01 (A; a)	0.62	79.6 (66.8 - 92.3) / (A; a)	2.13 (0.26 – 19.84) /0.27 (A; a)	0.55
		SOL	55.7 (35.2 - 76.2) / (A; a)	52.25 (0.91 - >10^4) /0.42 (B; a,b)	0.44	67.0 (57.5 - 76.5) / (A; a)	1.09 (0.13 – 12.25) /0.01 (A; a)	0.68
	2 days induced	MXF	61.2 (50.6 - 71.8) / (A; a)	7.13 (1.11 – 458.47) /0.89 (A; a)	0.76	73.1 (64.3 - 81.9) / (A; a)	0.73 (0.12 – 4.91) /0.09 (A; a)	0.62
		SOL	37.4 (21.9 - 52.9) / (B; a)	>10^4 (49.22 - >10^4) />80 (A; a,b)	0.44	59.5 (51.1 - 68.0) / (A,B; a)	1.47 (0.16 – 62.07) /0.01 (A; a)	0.63
	11 days naïve	MXF	49.9 (43.4 - 56.4) / (A; a)	>10^4 (5.37 - >10^4) />1250 (A; a)	0.75	18.7 (12.2 - 25) / (A; a)	>10^4 (>10^4 - >10^4) />1250 (A; a)	0.39
		SOL	42.2 (29.6 - 54.8) / (A; a,c)	>10^4 (6.27 - >10^4) />80 (A; a)	0.36	32.5 (14.9 - 50.1) / (B; a,c)	>10^4 (898.86 - >10^4) />80 (A; a)	0.23
	11 days induced	MXF	45.3 (38.7 - 51.9) / (A; a)	>10^4 (95.91 - >10^4) / >1250 (A; a)	0.77	22.4 (15.8 - 28.9) / (A; a)	>10^4 (>10^4 - >10^4) />1250 (A; a)	0.26
		SOL	35.7 (31.6 - 39.8) / (A,B; a)	>10^4 (>10^4 - >10^4) / >80 (A; a)	0.75	12.5 (3.3 - 21.8) / (A; a,c)	>10^4 (>10^4 - >10^4) />80 (A; a)	0.11
SAL	2 days naïve	MXF	90.3 (72.5 - 108.1) / (A; a)	16.84 (2.76 - 120.00) /2.11 (A; b)	0.82	77.8 (61.8 - 93.9) / (A; a,b)	2.45 (0.25 – 32.92) /0.31 (A; a)	0.83
		SOL	81.5 (73.3 - 89.7) / (A; b)	<10^{-4} (<10^{-4} – 0.07) /< 8x10^{-7} (B; b)	0.45	97.9 (89.7 - 106.1) / (A; b)	2.40 (0.92 – 6.35) /0.02 (A; a)	0.98
	2 days induced	MXF	89.9 (84.1 - 95.6) / (A; b)	0.05 (0.02 – 0.12) /0.01 (A; b)	0.97	72.1 (62.6 - 81.6) / (A; a,b)	0.02 (0.002 – 0.27) /0.01 (A; b)	0.76
		SOL	98.1 (83.6 - 112.6) / (A; b)	33.50 (10.60 – 114.34) /0.27 (B; a)	0.95	27.7 (16.2 - 39.2) / (B; b)	>10^4 (>10^4 - >10^4) />80 (B; b)	0.39
	11 days naïve	MXF	85.6 (77.7 - 93.5) / (A; b)	0.48 (0.16 – 1.51) /0.06 (A; b)	0.93	22.7 (9 - 36.5) / (A; a,b)	>10^4 (>10^4 - >10^4) />1250 (A; a)	0.29
		SOL	57.4 (49.5 - 65.3) / (B; a)	1.12 (0.03 - >10^4) /0.01 (A; a)	0.66	24.2 (10.9 - 37.4) / (A; a,c)	>10^4 (>10^4 - >10^4) />80 (A; a)	0.36
	11 days induced	MXF	52.3 (41 - 63.7) / (A; a)	34.43 (1.32 - >10^4) /4.30 (A; a,b)	0.72	28.7 (20.4 - 37) / (A,B; a)	>10^4 (>10^4 - >10^4) />1250 (A; a)	0.35
		SOL	47.4 (37.4 - 57.4) / (A; a)	>10^4 (0.05 - >10^4) / >80 (A; a,b)	0.74	43.6 (30.9 - 56.4) / (B; b)	>10^4 (0.02 - >10^4) />80 (A; a)	0.55
SAL+ZAN	2 days naïve	MXF	82.8 (73.7 - 92.0) / (A; a)	0.09 (0.03 – 0.30) /0.01(A; a)	0.94	80.7 (65.8 - 95.6) / (A; a)	9.6 (1.45 – 78.79) /1.20 (A; a)	0.84
		SOL	53.1 (32.6 - 73.4) / (A; a)	326.04 (2.19 - >10^4) /2.61 (B; b)	0.47	55.3 (37.1 - 73.5) / (A; a)	41.27 (0.39 - >10^4) /0.33 (A; a)	0.44
	2 days induced	MXF	64.4 (57.7 - 71.1) / (A; a)	1.50 (0.46 – 6.19) /0.19 (A; a)	0.94	63.8 (51.1 - 76.6) / (A; a)	33.41 (2.89 – 2465.01) /4.17 (A; a)	0.81
		SOL	39.6 (30.7 - 48.6) / (B; a)	>10^4 (>10^4 - >10^4) />80 (B; b)	0.68	23.6 (10.8 - 36.3) / (B; b)	>10^4 (>10^4 - >10^4) / >80 (B; b)	0.31
	11 days naïve	MXF	40.2 (22.5 - 57.9) / (A; a)	>10^4 (0.70 - >10^4) />1250 (A; a)	0.54	12.2 (4.7 - 19.7) / (A; a)	>10^4 (>10^4 - >10^4) />1250 (A; a)	0.42
		SOL	-0.6 (-3.3 - 2.1) / (B; b)	>10^4 (>10^4 - >10^4) >80 (A; a)	0.10	13.7 (5.7 - 21.6) / (A; a)	>10^4 (>10^4 - >10^4) />80 (A; a)	0.02
	11 days induced	MXF	41.3 (31 - 51.6) / (A; a)	>10^4 (9.12 - >10^4) />1250 (A; a)	0.64	17.3 (6 - 28.6) / (A,B; a)	>10^4 (>10^4 - >10^4) /> 1250 (A; a)	0.12
		SOL	4.7 (2.9 - 6.4) / (B; b)	>10^4 (>10^4 - >10^4) />80 (A; a)	0.10	6.5 (0.3 - 12.7) / (B; a)	>10^4 (>10^4 - >10^4) />80 (A; a)	0.12

SAL+IPR	2 days naïve	MXF	80.4 (63.8 - 97.0) / (A; a)	10.23 (0.95 – 143.45) /1.28 (A; a,b)	0.66	50.0 (37.8 - 62.4) / (A; b)	741.77 (0.75 - >10^4) /92.72 (A; a)	0.85
		SOL	87.6 (77.1 - 98.2) / (A; b)	0.05 (0.01 – 0.31) /0.01 (A; a)	0.89	86.1 (69.1 - 103.0) / (B; a,b)	8.37 (1.12 – 74.85) /0.07 (A; a)	0.85
	2 days induced	MXF	94.7 (86.4 - 103.0) / (A; b)	0.01 (0.001 – 0.08) /0.001 (A; a)	0.76	102.6 (81.6 - 123.6) / (A; b)	1.56 (0.11 – 20.36) /0.19 (A; a,b)	0.82
		SOL	104. 8 (85.4 - 124.1) / (A; b)	35.58 (9.16 – 154.31) /0.28 (B; a)	0.93	9.3 (2.6 -16.0) / (B; c)	>10^4 (>10^4 - >10^4) />80 (B; b)	0.58
	11 days naïve	MXF	75.9 (64.8 - 86.9) / (A; b)	1.29 (0.29 – 6.98) /0.16 (A; a)	0.83	49.9 (30 - 69.8) / (A; b)	>10^4 (0.14 - >10^4) />1250 (A; a)	0.34
		SOL	25.6 (13.0 - 38.2) / (B; c)	>10^4 (>10^4 - >10^4) />80 (B; a)	0.60	66.3 (55.2 - 77.5) / (A; b)	3.12 (0.53 – 33.76) /0.02 (A; b)	0.91
	11 days induced	MXF	90.0 (75.3 - 104.6) / (A; b)	0.91 (0.13 – 6.82) /0.11 (A; b,c)	0.72	61.1 (43.8 - 78.3) / (A; b)	4.89 (0.06 - >10^4) /0.61 (A; a,b)	0.38
		SOL	45.2 (34.0 - 56.3) / (B; a)	>10^4 (>10^{-3} - >10^4) / >80 (B; a,b)	0.56	1.2 (-2.4 – 4.8) / (B; a)	>10^4 (>10^4 - >10^4) />80 (B; a)	0.72
IPR	2 days naïve	MXF	95.0 (78.7 - 111.2) / (A; a)	5.31 (0.68 – 40.96) /0.66 (A; a,b)	0.86	74.7 (54.3 - 95.2) / (A; a,b)	18.98 (1.28 – 806.28) /2.37 (A; a)	0.77
		SOL	91.1 (79.0 - 103.1) / (A; b)	0.11 (0.02 – 0.67) /0.01 (A; a)	0.89	81.9 (57.4 - 106.3) / (A; a,b)	73.65 (6.94 – 1800.73) /0.59 (A; a)	0.81
	2 days induced	MXF	97.0 (90.1 - 103.9) / (A; b)	<10^{-4} (<10^{-4} – 2.16) /<0.0125 (A; a)	0.94	98.6 (82.9 - 114.3) / (A; b)	0.16 (0.02 – 1.46) /0.02 (A; a,b)	0.85
		SOL	94.0 (80.2 - 107.9) / (A; b)	89.75 (33.68 – 262.83) /0.72 (B; a)	0.96	35.1 (19.7 - 50.4) / (C; b)	>10^4 (26.81 - >10^4) / >80 (B; a,b)	0.48
	11 days naïve	MXF	104.0 (88.9 - 119) / (A; c)	0.37 (0.07 – 1.89) /0.05 (A; a)	0.91	94.5 (83 - 106) / (A; c)	0.001 (10^{-4} - 0.006) /10^{-4} (A; b)	0.91
		SOL	59.1 (46.6 - 71.6) / (C; a)	1.17 (0.01 - >10^4) /0.01 (A; a)	0.56	44.1 (33.7 - 54.5) / (B; c)	>10^4 (0.01 - >10^4) />80 (B; a,b)	0.40
	11 days induced	MXF	99.0 (89.7 - 108.3) / (A; b)	0.04 (0.01 – 0.19) /0.01 (A; c)	0.91	100.0 (96.2 - 103.9) / (A; c)	0.01 (0.004 – 0.018) /0.01 (A; b)	0.98
		SOL	66.9 (52.4 - 81.3) / (B; c)	3.12 (0.15 – 212.09) /0.02 (A; b)	0.75	24.6 (14.3 - 34.9) / (B; b,c)	>10^4 (>10^4 - >10^4) />80 (B; a)	0.74

[a] Calculated based on sigmoidal regressions with a Hill coefficient of 1

[b] Decrease in viability and matrix thickness from the original values obtained under control conditions (growth without antibiotic) as extrapolated for an infinitely large concentration of antibiotic (means with 95% confidence intervals).

[c] Statistical analysis: One-way ANOVA with Tukey's post-test for multiple comparisons between different culture media for each drug and type of biofilm (small letters) and between antibiotics for each type of biofilm (caps letters). Values with different letters are significantly different from each other ($p<0.05$).

Table S4. Pertinent regression parameters[a] with 95% confidence intervals and statistical analysis[c] for strain R6

Media	Biofilm models	Antibiotics	Effect on viability within the matrix			Effect on biofilm thickness		
			E_{max}[b] % loss of viability (CI at 95%)	Concentration (X MIC - mg/L) yielding 50% reduction	R^2	E_{max}[b] % loss of matrix (CI at 95%)	Concentration (X MIC- mg/L) yielding 50% reduction	R^2
CTRL	2 days naïve	MXF	86.54 (76.69 - 96.39) / (A; a)	2.49 (0.75 - 8.62)/ 0.16 (A; a)	0.76	68.63 (59.80 - 77.46)/ (A; a)	1.05 (0.16 - 8.64) / 0.07 (A; a)	0.54
		SOL	50.45 (37.32 - 55.95) / (B; a)	1227.22 (7.73 - >10^4)/ 4.91 (A; a)	0.52	51.10 (14.04 - 88.16)/ (A; a,b)	3763.55 (0.19 - >10^4) / 15.05 (A; a)	0.06
	2 days induced	MXF	46.32 (29.61 - 63.03) / (A; a)	>10^4 (4.86 - >10^4)/ > 640 (A; a)	0.39	49.32 (29.53 - 69.10)/ (A; a)	>10^4 (1.45 - >10^4) / >640 (A; a)	0.26
		SOL	42.73 (22.01 - 63.46) / (A; a)	>10^4 (2,89 - >10^4)/ >40 (A; a,b)	0.26	24.85 (11.78 - 37.93)/ (A; a)	>10^4 (>10^4 - >10^4) / >40 (A; a)	0.09
	11 days naïve	MXF	30.82 (18.15 - 43.48) / (A; a)	>10^4 (>10^4 - >10^4)/ >640 (A; a)	0.43	23.67 (17.96 - 29.39)/ (A; a)	>10^4 (>10^4 - >10^4) / >640 (A; a)	0.36
		SOL	39.77 (20.85 - 58.70) / (A; a)	>10^4 (12.14 - >10^4)/ >40 (A; a,b)	0.25	20.84 (9.53 - 32.15)/ (A; a)	>10^4 (>10^4 - >10^4) / >40 (A; a)	0.08
	11 days induced	MXF	48.73 (37.20 - 60.25) / (A; a)	>10^4 (7.41 - >10^4)/ >640 (A; a,b)	0.64	9.66 (5.64 - 13.68)/ (A; a)	>10^4 (>10^4 - >10^4) / >640 (A; a)	0.28
		SOL	46.74 (37.52 - 55.95) / (A; a)	>10^4 (111.53 - >10^4) / >40 (A; a)	0.72	15.05 (6.88 - 23.22) / (A,B; a)	>10^4 (>10^4 - >10^4) / >40 (A; a)	0.06
SAL	2 days naïve	MXF	94.98 (76.27 - 113.69) / (A; a)	1.05 (0.08 - 13.32) / 0.07 (A; a)	0.70	80.44 (55.86 - 105.02) / (A; a,b)	2.89 (0.22 - 99.33) / 0.18 (A; a)	0.64
		SOL	98.46 (92.76 - 104.15) / (A; b)	0.17 (0.08 - 0.34) / 0.01 (A; b)	0.98	93.29 (69.09 - 117.48) / (A; a,b)	19.15 (1.25 - 353.74) / 0.08 (A; a)	0.79
	2 days induced	MXF	86.58 (76.95 - 96.21) / (A; b)	0.11 (0.02 - 0.51) / 0.01 (A; b)	0.82	90.91 (62.64 - 119.19) / (A; a,b)	9.08 (0.93 - 156.20) / 0.58 (A,B; a)	0.65
		SOL	97.45 (83.70 - 111.21) / (A; b)	35.34 (12.17 - 110.71) / 0.14 (B; a)	0.96	33.26 (25.98 - 40.54) / (B; a)	>10^4 (>10^4 - >10^4) / >40 (B; a)	0.82
	11 days naïve	MXF	95.45 (83.44 - 107.47) / (A; b)	5.13 (1.57 - 17.63) / 0.33 (A; b)	0.95	48.05 (25.23 - 70.87) / (A; a)	>10^4 (0.18 - >10^4) / >640 (A; a)	0.53
		SOL	66.38 (50.78 - 81.97) / (B; b)	1.88 (0.06 - 530.17) / 0.01 (A; b)	0.68	55.61 (45.63 - 65.59) / (A; b)	2.68 (0.21 - >10^4) / 0.01 (A; a)	0.88
	11 days induced	MXF	98.67 (83.52 - 113.83) / (A; b)	5.01 (1.32 - 20.47) / 0.32 (A; b)	0.94	15.30 (-2.40 - 33.02) / (A; a,b)	>10^4 (>10^4 - >10^4) / >640 (A; a)	0.04
		SOL	49.14 (36.91 - 61.36) / (B;a)	>10^4 (0.002 - >10^4) / >40 (A; a)	0.55	21.79 (12.61 - 30.96) / (A; a)	>10^4 (>10^4 - >10^4) / >40 (A; a)	0.40
SAL+ZAN	2 days naïve	MXF	93.06 (76.68 - 109.45) / (A; a)	1.34 (0.13 - 13.24) / 0.08 (A,B; a)	0.75	81.87 (71.57 - 92.16) / (A; a,b)	1.86 (0.44 - 8.59) / 0.12 (A; a)	0.90
		SOL	93.97 (82.36 - 105.58) / (A; b)	0.25 (0.04 - 1.55) / 0.01 (B; b)	0.83	49.36 (26.16 - 72.57) / (B; a)	>10^4 (11.97 - >10^4) / >40 (B; a)	0.41
	2 days induced	MXF	52.52 (33.98 - 71.06) / (A,B; a)	128.61 (1.35 - >10^4)/ 8.23 (A,B; a)	0.56	60.86 (35.60 - 86.12) / (A; a,b)	82.12 (0.99 - >10^4) / 5.25 (A; a)	0.46
		SOL	34.11 (20.95 - 47.27) / (B; a)	>10^4 (>10^4 - >10^4) / > 40 (B; b)	0.33	2.82 (-4.40 - 10.00) / (C; b)	>10^4 (>10^4 - >10^4) / >40 (A; a)	0.12
	11 days naïve	MXF	18.94 (10.19 - 27.69) / (A; a)	>10^4 (>10^4 - >10^4)/ >640 (A; a)	0.32	15.64 (3.68 - 27.6) / (A; a)	>10^4 (>10^4 - >10^4) / >640 (A ; a)	0.05
		SOL	5.60 (4.13 - 7.06) / (B; c)	>10^4 (>10^4 - >10^4)/ >40 (A; a)	0.48	14.28 (6.47 - 22.10) / (A; a)	>10^4 (>10^4 - >10^4) / >40 (A ; a)	0.19
	11 days induced	MXF	38.32 (32.64 - 43.99) / (A; a)	>10^4 (>10^4 - >10^4) / >640 (A; a)	0.88	4.94 (-0.60 - 10.44) / (A; a)	>10^4 (>10^4 - >10^4) / >640 (A ; a)	0.05
		SOL	6.31 (2.42 - 10.20) / (B; b)	>10^4 (>10^4 - >10^4) / >40 (A; a)	0.05	1.16 (-11.40 - 13.71) / (A; b)	>10^4 (>10^4 - >10^4) / >40 (A ; a)	0.02

SAL+IPR	2 days naïve	MXF	100.98 (87.74 - 114.22) / (A; a)	2.27 (0.47 - 10.92) / 0.14 (A; a)	0.92	93.82 (74.02 - 113.61) / (A; a,b)	2.74 (0.34 - 25.56) / 0.18 (A; a)	0.73
		SOL	99.12 (85.77 - 112.48) / (A; b)	0.63 (0.13 - 3.11) / 0.01 (A; b)	0.91	101.28 (78.88 - 123.68) / (A; b)	6.84 (0.13 - 136.23) / 0.03 (A; a)	0.77
	2 days induced	MXF	84.78 (61.24 - 108.32) / (A; a,b)	1.00 (0.04 - 37.23) / 0.06 (A; a,b)	0.51	99.04 (69.92 - 128.15) / (A; a,b)	2.97 ($<10^{-4}$ - 170.97) / 0.19 (A; a,b)	0.37
		SOL	102.70 (85.32 - 120.08) / (A; b)	29.98 (8.18 - 120.76) / 0.12 (A; a)	0.94	50.69 (38.16 - 63.22) / (C; c)	618.23 (0.75 - $>10^4$) / 2.47 (A,B; a)	0.48
	11 days naïve	MXF	102.92 (92.64 - 113.2) / (A; b)	2.31 (0.74 - 7.24) / 0.15 (A; b)	0.91	87.71 (78.51 - 96.91) / (A; b)	0.001 ($<10^{-4}$ - 0.01) / $<10^{-4}$ (A ; b)	0.65
		SOL	35.35 (21.67 - 49.04) / (B; a)	$>10^4$ ($>10^4$ - $>10^4$) / >40 (B; a)	0.50	27.24 (19.29 - 35.19) / (B; a)	$>10^4$ ($>10^4$ - $>10^4$) / >40 (C ; a)	0.21
	11 days induced	MXF	90.50 (72.73 - 108.27) /(A,B; b)	3.89 (0.58 - 30.24) / 0.25 (A; b)	0.83	30.73 (22.28 - 39.17) / (A; b)	$>10^4$ ($>10^4$ - $>10^4$) / >640 (A ; a)	0.35
		SOL	69.26 (53.66 - 84.86) / (B; a)	2.05 (0.13 - 85.06) / 0.01 (A; a)	0.77	41.39 (24.94 - 57.85) / (A; c)	$>10^4$ (5.52 - $>10^4$) / >40 (A ; a)	0.62
IPR	2 days naïve	MXF	94.96 (78.73 - 111.18) / (A; a)	5.31 (0.68 - 40.96) / 0.34 (A,B; a)	0.86	94.96 (81.83 - 106.76) / (A; b)	3.12 (0.89 - 11.59) / 0.20 (A; a)	0.94
		SOL	99.85 (90.72 - 108.98) / (A; b)	0.26 (0.09 - 0.74) / 0.01 (B; b)	0.96	90.30 (58.36 - 122.25) / (A; a,b)	184.48 (20.91 - 4230.31) /0.74 (B; a)	0.83
	2 days induced	MXF	103.95 (89.58 - 118.32) / (A; b)	20.94 (5.64 - 80.14)/ 1.34 (A; a)	0.94	103.95 (86.31 - 119.67) / (A; b)	$<10^{-4}$ ($<10^{-4}$ - $<10^{-4}$) / >640 (A; b)	0.43
		SOL	99.94 (82.67 - 117.22) / (A; b)	37.79 (10.38 - 152.93) / 0.15 (A; a)	0.94	45.46 (30.81 - 60.11) / (B; c)	$>10^4$ (0.001 - $>10^4$) / >40 (A,B ; a)	0.19
	11 days naïve	MXF	103.98 (88.94 - 119.01) / (A; b)	0.37 (0.07 - 1.89) / 0.02 (A; b)	0.91	103.98 (76.94 - 102.90) / (A; b)	0.003 (0.0003 - 0.022) /0.01 (A ; b)	0.87
		SOL	54.62 (38.58 - 70.66) /(B; a,b)	2.61 (0.0004 - $>10^4$) / 0.01 (A; a)	0.28	20.23 (15.33 - 25.13) / (B; a)	$>10^4$ ($>10^4$ - $>10^4$) />40 (C ; a)	0.61
	11 days induced	MXF	100.42 (90.60 - 110.23) / (A; b)	0.11 (0.03 - 0.47) / 0.01 (A; c)	0.93	100.42 (93.49 - 112.61) / (A; c)	0.04 (0.01 - 0.13) / 0.01 (A ; b)	0.95
		SOL	51.21 (35.91 - 66.51) / (C; a)	3.51 (0.003 - $>10^4$) / 0.01 (A; a)	0.50	41.31 (29.44 - 53.17) / (B; c)	$>10^4$ (0.05 - $>10^4$) / >40 (A,B ; a)	0.50

[a] Calculated based on sigmoidal regressions with a Hill coefficient of 1

[b] Decrease in viability and matrix thickness from the original values obtained under control conditions (growth without antibiotic) as extrapolated for an infinitely large concentration of antibiotic (means with 95% confidence intervals).

[c] Statistical analysis: One-way ANOVA with Tukey's post-test for multiple comparisons between different culture media for each drug and type of biofilm (small letters) and between antibiotics for each type of biofilm (caps letters). Values with different letters are significantly different from each other ($p<0.05$).

Table S5. Pertinent regression parameters[a] with 95% confidence intervals and statistical analysis[c] for strain N6

Media	Biofilm models	Antibiotics	Effect on viability within the matrix			Effect on biofilm thickness		
			E_{max}[b] % loss of viability (CI at 95%)	Concentration (X MIC - mg/L) yielding 50% reduction	R^2	E_{max}[b] % loss of matrix (CI at 95%)	Concentration (X MIC- mg/L) yielding 50% reduction	R^2
CTRL	2 days naïve	MXF	85.70 (73.95-97.45) / (A; a)	9.39 (<10^{-4} – 34.68) /0.60 (A; a)	0.84	76.83 (65.55-88.11) / (A; a,b)	1.27 (0.27 – 7.11) /0.90 (A; a,b)	0.77
		SOL	102.31 (95.85-108.77) / (A; a)	4.08 (2.25 – 7.45) /0.02 (A; a)	0.98	96.48 (77.13-115.82) / (A; a)	15.66 (1.29 – 173.33) /0.06 (A; a)	0.73
	2 days induced	MXF	63.36 (53.48-73.24) / (A; a)	13.64 (2.59 – 156.01) /0.87 (A; a)	0.79	76.51 (22.93-130.08) / (A; a)	551.89 (18.40 - >10^4) /35.32 (A; a)	0.63
		SOL	53.62 (45.35-61.89) / (A; a)	92.89 (8.45 – 10.75) /0.37 (A; a)	0.83	48.14 (39.44-56.84) / (A; a)	>10^4 (>10^{-4} - >10^4) />40 (A; a)	0.61
	11 days naïve	MXF	54.36 (41.90-66.82) / (A; a)	87.57 (5.15 - >10^4) /5.60 (A; a)	0.71	48.20 (37.77-58.63) / (A; a)	>10^4 (1.09 - >10^4) />640 (A; a)	0.54
		SOL	50.40 (31.43-69.37) / (A; a)	2106.49 (3.60 - >10^4) /8.43 (A; a)	0.54	80.09 (63.90-96.28) / (B; a)	15.14 (2.40 – 127.56) /0.06 (A; a)	0.82
	11 days induced	MXF	43.84 (29.99-57.69) / (A; a)	>10^4 (5.26 - >10^4) />640 (A; a)	0.50	39.52 (10.20-68.83) / (A; a)	>10^4 (0.11 - >10^4) />640 (A; a)	0.31
		SOL	24.35 (12.20-36.50) / (A; a)	>10^4 (>10^{-4} - >10^4) />40 (A; a)	0.49	43.41 (33.78-53.05) / (A; a)	>10^4 (0.01 - >10^4) />40 (A; a)	0.47
SAL	2 days naïve	MXF	91.95 (80.78-103.12) / (A; a)	6.26 (1.98 – 20.81) /0.40 (A; a)	0.92	89.97 (78.92-101.03) / (A; b)	0.40 (0.08 – 2.05) /0.03 (A; b)	0.86
		SOL	93.64 (88.20-99.08) / (A; a)	3.97 (2.23 – 7.13) /0.02 (A; a)	0.98	76.77 (68.77-84.77) / (B; a)	0.001 (<10^{-4} – 0.02) / <10^{-4} (B; b)	0.61
	2 days induced	MXF	63.88 (53.86-73.90) / (A; a)	20.44 (3.66 – 233.56) /1.31 (A; a)	0.85	32.80 (22.27-43.34) / (A; a)	>10^4 (>10^4 - >10^4) />640 (A; a)	0.65
		SOL	55.87 (38.85-72.89) / (A; a)	92.11 (2.24 - >10^4) /0.37 (A; a)	0.56	40.95 (11.47-70.43) / (A; a)	>10^4 (1.74 - >10^4) />40 (A; a)	0.29
	11 days naïve	MXF	59.50 (46.37-72.63) / (A; a)	14.70 (1.24 - >10^4) /0.94 (A; a)	0.78	49.12 (33.66-64.58) / (A; a)	>10^4 (0.09 - >10^4) />640 (A; a)	0.53
		SOL	36.23 (14.36-58.10) / (A; a,b)	>10^4 (0.96 - >10^4) />40 (A; a)	0.40	4.55 (-2.80-11.89) / (B; b)	>10^4 (>10^4 - >10^4) />40 (A; b)	0.01
	11 days induced	MXF	49.81 (26.68-72.93) / (A; a)	>10^4 (4.27 - >10^4) />640 (A; a)	0.49	57.12 (44.02-70.22) / (A; a)	4.65 (0.21 - >10^4) /0.30 (A; a)	0.67
		SOL	32.42 (12.61-52.24) / (A; a,b)	>10^4 (1.20 - >10^4) />40 (A; a)	0.22	22.08 (9.02-35.15) / (B; a,b)	>10^4 (>10^4 - >10^4) />40 (A; a)	0.09
SAL+ZAN	2 days naïve	MXF	78.76 (38.67-118.85) / (A; a)	149.70 (4.86 - >10^4) /9.58 (A; a)	0.47	55.99 (46.40-65.59) / (A,B; a)	83.75 (6.98 - >10^4) /5.36 (A; a)	0.65
		SOL	90.99 (70.17-111.80) / (A; a)	14.61 (1.17 – 198.13) /0.06 (A; a)	0.87	44.17 (36.51-51.84) / (B; b)	>10^4 (0.23 - >10^4) />40 (A; a)	0.43
	2 days induced	MXF	28.91 (18.35-39.47) / (A; b)	>10^4 (>10^4 - >10^4) />640 (A; b)	0.65	51.37 (34.86-67.87) / (A; a)	181.30 (1.59 - >10^4) /11.60 (A; a)	0.63
		SOL	43.45 (32.96-53.94) / (A; a)	>10^4 (0.69 - >10^4) />40 (A; a)	0.51	45.98 (38.04-53.91) / (A; a)	>10^4 (0.41 - >10^4) />40 (A; a)	0.75
	11 days naïve	MXF	50.46 (43.63-57.30) / (A; a)	828.99 (22.37 - >10^4) /53.06 (A; a)	0.91	44.90 (29.59-60.21) / (A; a)	>10^4 (4.64 - >10^4) />640 (A; a)	0.45
		SOL	19.85 (15.09-24.62) / (B; b)	>10^4 (>10^4 - >10^4) />40 (A; a)	0.75	39.44 (16.45-62.43) / (A,B; a)	>10^4 (>10^4 - >10^4) />40 (A; b)	0.33
	11 days induced	MXF	36.25 (28.02-44.48) / (A; a)	>10^4 (>10^4 - >10^4) />640 (A; a)	0.74	36.06 (-19.00-91.11) / (A; a)	>10^4 (1.48 - >10^4) />640 (A; a)	0.21
		SOL	10.94 (6.50-15.39) / (B; a)	>10^4 (>10^{-4} - >10^4) />40 (A; a)	0.25	9.28 (-4.20-22.81) / (A; b)	>10^4 (>10^4 - >10^4) />40 (A; a)	0.04

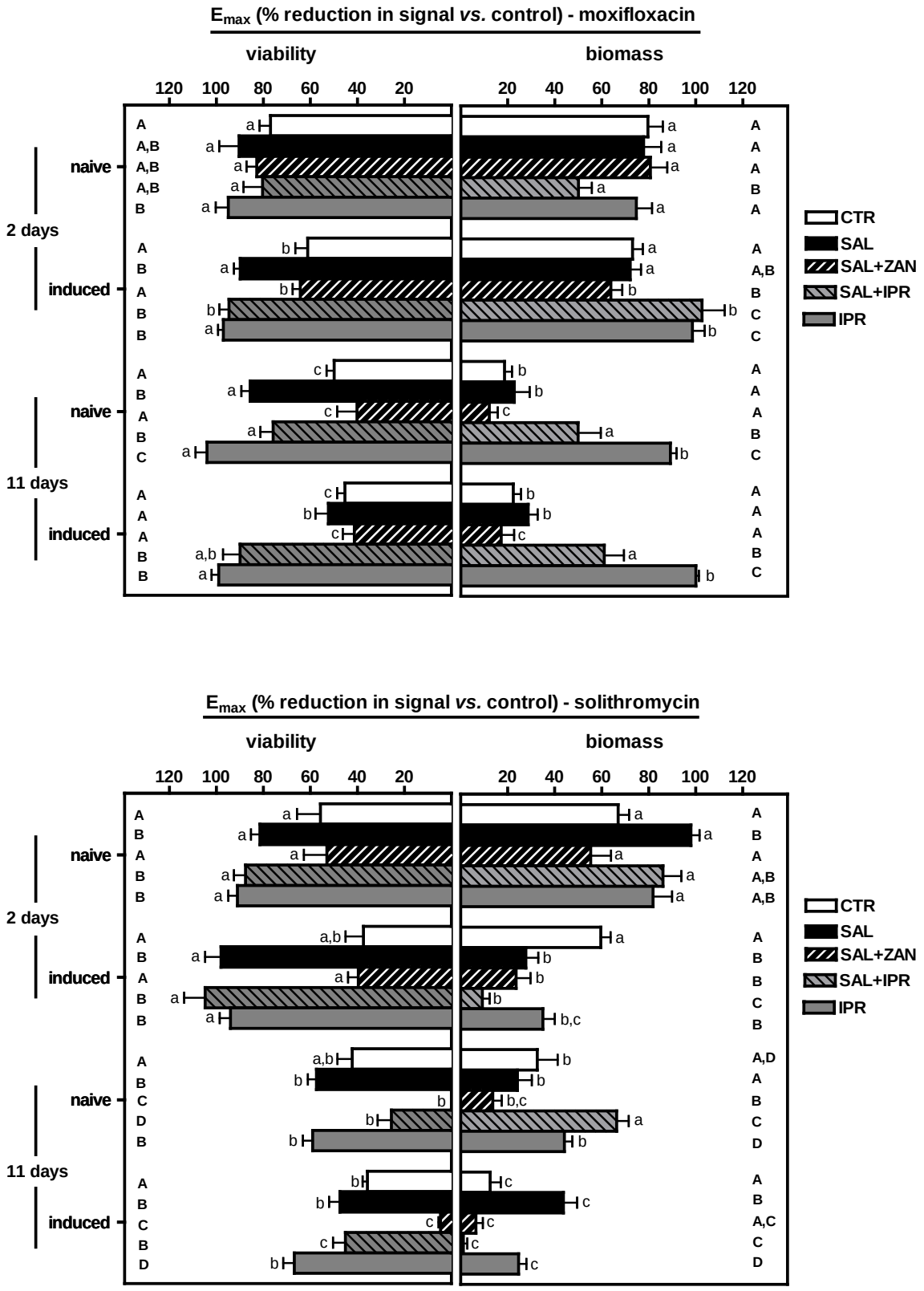
SAL+IPR	2 days naïve	MXF	102.39 (96.07-108.71) / (A; a)	0.48 (0.23 – 0.98) /0.03 (A; b)	0.97	84.46 (73.06-95.87) / (A; b)	0.11 (0.01 – 0.94) /0.01 (A; a,b)	0.77
		SOL	102.20 (96.67-107.72) / (A; a)	1.86 (1.08 – 3.23) /0.01 (A; a)	0.98	71.67 (56.18-87.16) / (A; a)	7.12 (0.18 – 439.69) /0.03 (A; a)	0.56
	2 days induced	MXF	78.86 (58.70-99.02) / (A; a)	5.47 (0.26 – 192.61) /0.35 (A; a)	0.61	42.00 (31.13-52.87) / (A; a)	$>10^4$ (30.38 - $>10^4$) />640 (A; a)	0.72
		SOL	54.30 (47.31-61.28) / (A; a)	26.01 (2.69 - $>10^4$) /0.10 (A; a)	0.84	43.09 (36.69-49.50) / (A; a)	$>10^4$ ($>10^{-4}$ - $>10^4$) />40 (A; a)	0.52
	11 days naïve	MXF	61.58 (43.19-79.97) / (A; a)	39.34 (1.76 - $>10^4$) /2.52 (A; a)	0.62	29.89 (13.82-45.97) / (A; a,b)	$>10^4$ ($>10^4$ - $>10^4$) />640 (A; a)	0.01
		SOL	39.84 (22.64-57.04) / (A; a,b)	$>10^4$ (0.19 - $>10^4$) />40 (A; a)	0.36	10.46 (3.68-17.24) / (A,B; b)	$>10^4$ ($>10^4$ - $>10^4$) />40 (A; b)	0.12
	11 days induced	MXF	93.85 (81.88-105.81) / (A; b)	0.34 (0.05 – 2.11) /0.02 (A; b)	0.84	43.87 (27.49-60.24) / (A; a)	$>10^4$ (0.05 - $>10^4$) />640 (A; a)	0.29
		SOL	61.39 (48.40-74.38) / (B; b)	1.27 (0.03 - $>10^4$) /0.01 (A; a)	0.57	44.42 (32.92-55.91) / (A; a)	$>10^4$ (0.01 - $>10^4$) />40 (A; a)	0.23
IPR	2 days naïve	MXF	100.66 (91.91-109.41) / (A; a)	1.62 (0.62 – 4.29) /0.10 (A; a,b)	0.94	93.34 (86.12-100.57) / (A; b)	0.07 (0.02 – 0.22) /0.01 (A; a,b)	0.92
		SOL	96.22 (89.97-102.47) / (A; a)	2.47 (1.26 – 4.88) /0.01 (A; a)	0.97	83.27 (71.99-94.55) / (A; a)	0.004 ($<10^{-4}$ – 0.02) /$<10^{-4}$ (A; b)	0.53
	2 days induced	MXF	59.64 (55.68-63.60) / (A; a)	46.39 (20.29 – 127.36) /2.97 (A; a)	0.97	31.81 (19.79-43.83) / (A; a)	$>10^4$ ($>10^4$ - $>10^4$) />640 (A; a)	0.49
		SOL	46.20 (35.09-57.31) / (B; a)	$>10^4$ (0.87 - $>10^4$) />40 (A; a)	0.60	36.32 (17.49-55.15) / (A; a)	$>10^4$ (0.05 - $>10^4$) />40 (A; a)	0.16
	11 days naïve	MXF	69.17 (57.91-80.43) / (A; a)	9.98 (1.47 – 98.93) /0.64 (A; a)	0.82	21.39 (13.46-29.32) / (A; b)	$>10^4$ ($>10^4$ - $>10^4$) />640 (A; a)	0.23
		SOL	27.75 (16.14-39.35) / (B; a,b)	$>10^4$ ($>10^{-4}$ - $>10^4$) />40 (A; a)	0.26	11.13 (4.98-17.28) / (A; b)	$>10^4$ ($>10^4$ - $>10^4$) />40 (A; b)	0.48
	11 days induced	MXF	87.19 (74.75-99.63) / (A; b)	0.17 (0.02 – 1.19) /0.01 (A; b)	0.80	42.87 (32.39-53.34) / (A; a)	$>10^4$ (1.03 - $>10^4$) />640 (A; a)	0.46
		SOL	40.40 (27.23-53.57) / (B; a,b)	$>10^4$ (0.64 - $>10^4$) />40 (A; a)	0.46	25.60 (20.44-30.76) / (A; b)	$>10^4$ ($>10^4$ - $>10^4$) />40 (A; a)	0.53

[a] Calculated based on sigmoidal regressions with a Hill coefficient of 1

[b] Decrease in viability and matrix thickness from the original values obtained under control conditions (growth without antibiotic) as extrapolated for an infinitely large concentration of antibiotic (means with 95% confidence intervals).

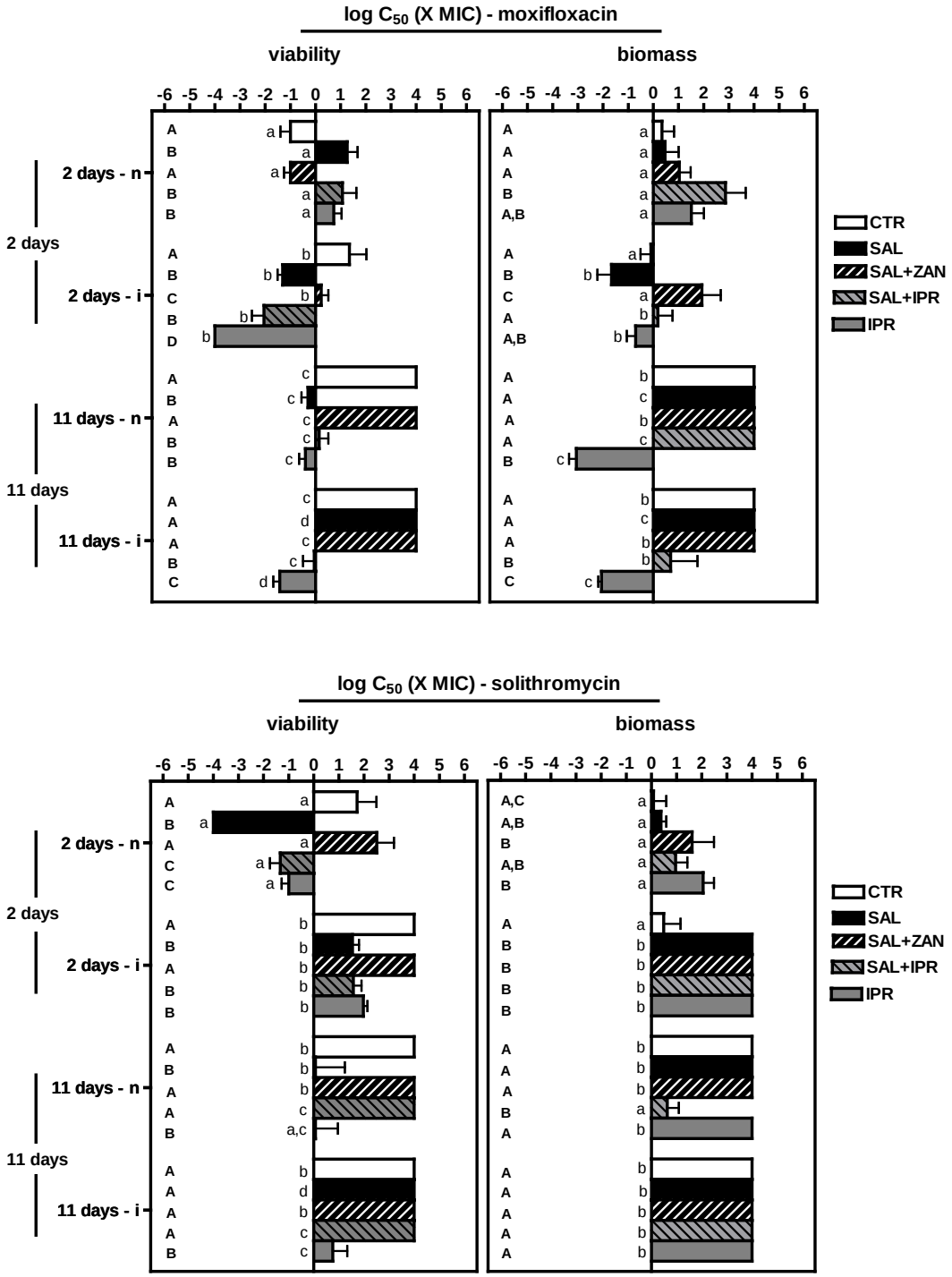
[c] Statistical analysis: One-way ANOVA with Tukey's post-test for multiple comparisons between different culture media for each drug and type of biofilm (small letters) and between antibiotics for each type of biofilm (caps letters). Values with different letters are significantly different from each other ($p<0.05$).

Figure S1


E$_{max}$ (% reduction in signal *vs.* control) - moxifloxacin
viability
biomass
120 100 80 60 40 20
20 40 60 80 100 120
2 days
11 days
naive
induced
CTR
SAL
SAL+ZAN
SAL+IPR
IPR
E$_{max}$ (% reduction in signal *vs.* control) - solithromycin
viability
biomass
120 100 80 60 40 20
20 40 60 80 100 120
2 days
11 days
naive
induced
CTR
SAL
SAL+ZAN
SAL+IPR
IPR

Caption to Figure S1: Comparison of antibiotic maximal efficacies (E_{max}) expressed as percentages reduction in resorufin fluorescence (viability; left panels) or crystal violet absorbance (biomass; right panels) as compared to controls (no antibiotic added) for 2-days and 11-days old naive and induced biofilms of strain ATCC49619. Top panels: moxifloxacin; bottom panels, solithromycin. Values were calculated as means ± SEM of 2-8 independent experiments performed each in quadruplicate (when not visible, the bars are smaller than the size of the symbols), using the Hill equation of the concentration-response curves; see also Table S3 in this Supplementary Material for numerical values). Statistical analyses: one-way ANOVA with Tukey's post-test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Small letters: comparison between different types of biofilms for each growth medium; caps letters: comparison between the different growth media for each type of biofilm.

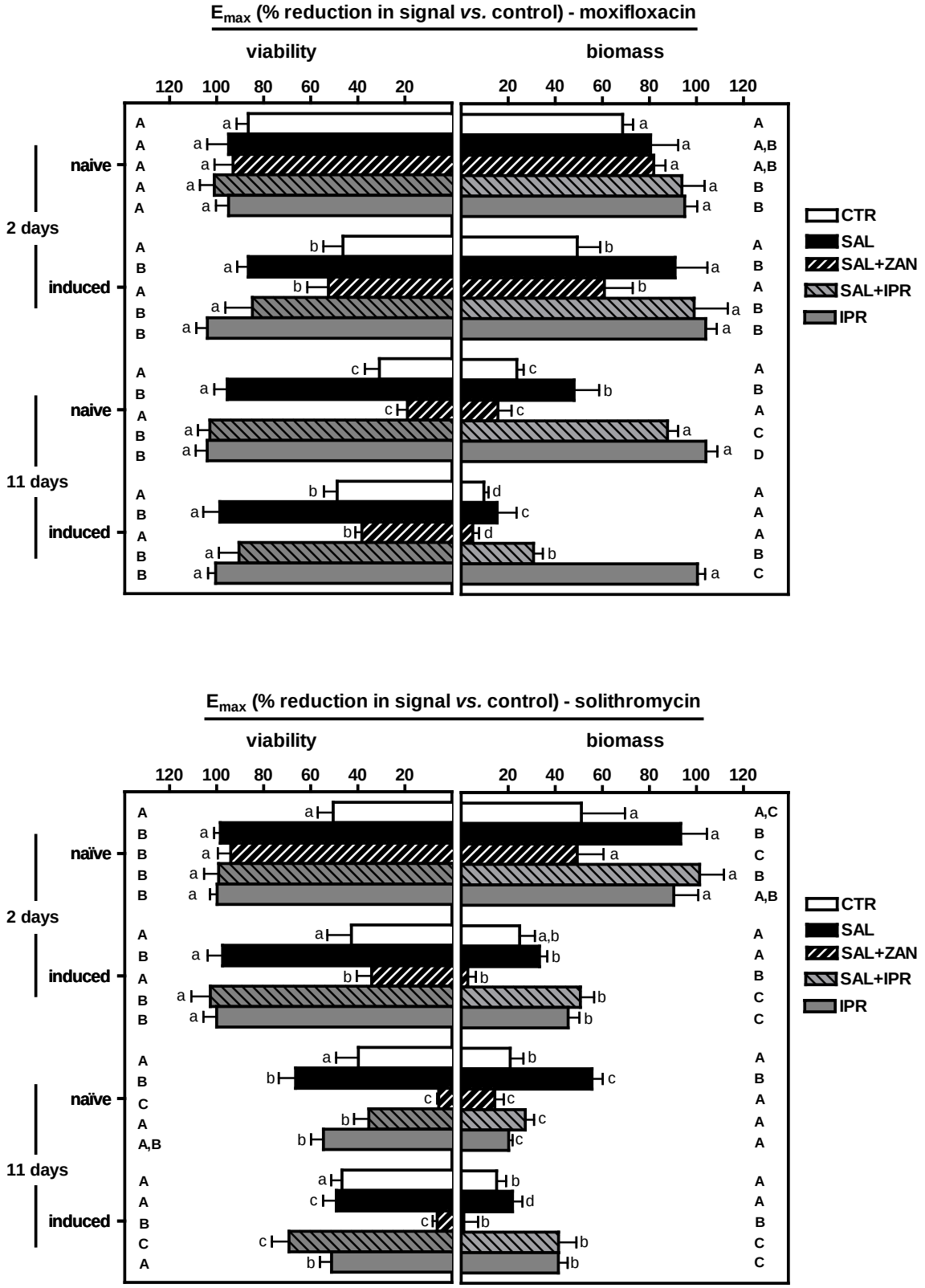
Figure S2



Caption to Figure S2: Comparison of antibiotic maximal relative potencies (C_{50}) expressed in multiples of the MIC towards viability (left panels) or biomass (right panels) for 2-days and 11-days old naive (n) and induced (i) biofilms of strain ATCC49619. Top panels: moxifloxacin; bottom panels, solithromycin. Values were calculated as means ± SD of 2-8

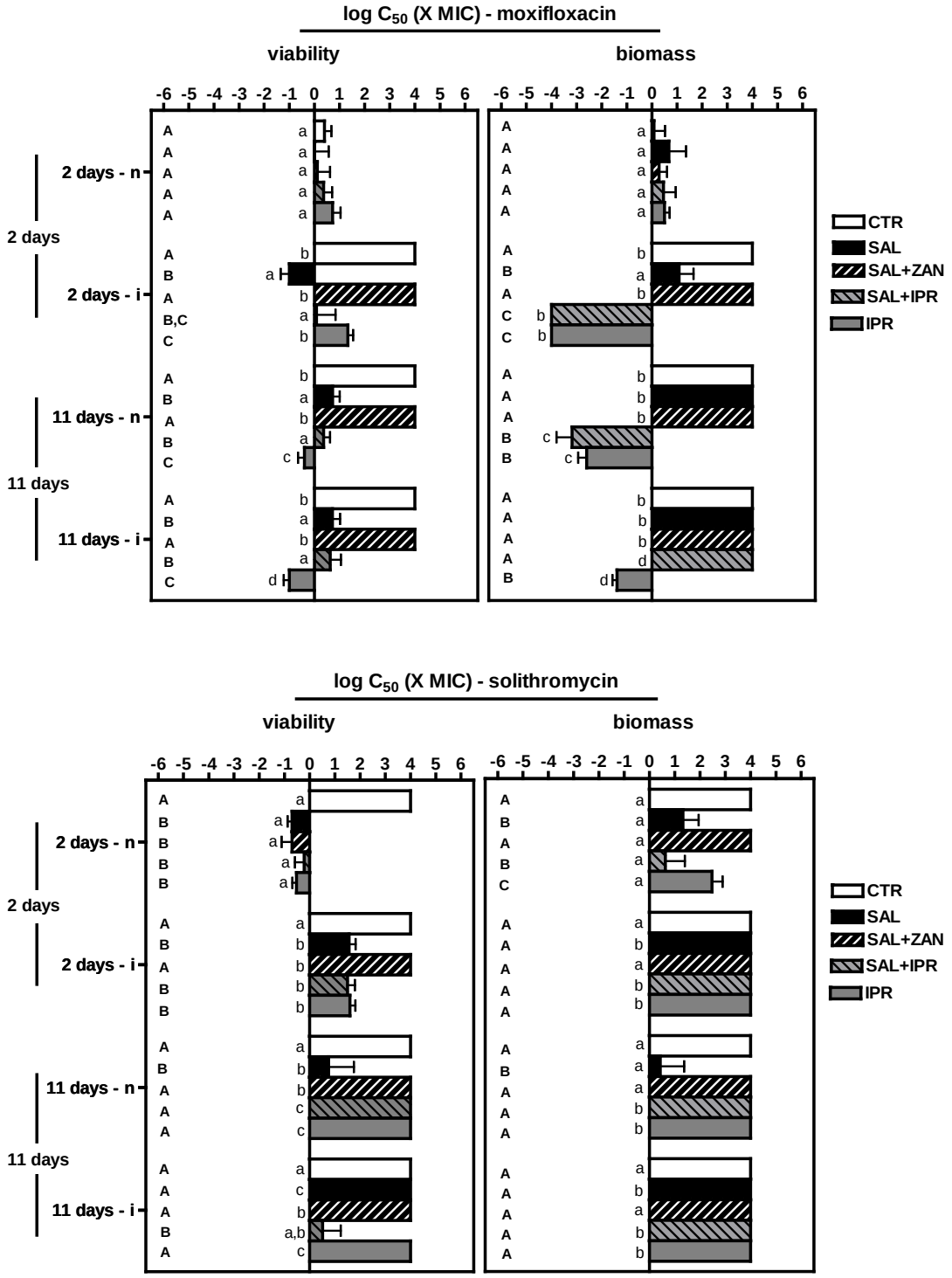
independent experiments performed in quadruplicates (when not visible, the bars are smaller than the size of the symbols), using the Hill equation of the concentration-response curves; see also Table S3 in this Supplementary Material for numerical values). Statistical analyses: one-way ANOVA with Tukey post-test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Small letters: comparison between different types of biofilms for each growth medium; caps letters: comparison between the different growth media for each type of biofilm.

Figure S3


E$_{max}$ (% reduction in signal *vs.* control) - moxifloxacin
viability
biomass
120 100 80 60 40 20
20 40 60 80 100 120
2 days
naive
induced
11 days
naive
induced
CTR
SAL
SAL+ZAN
SAL+IPR
IPR
E$_{max}$ (% reduction in signal *vs.* control) - solithromycin
viability
biomass
2 days
naïve
induced
11 days
naïve
induced
CTR
SAL
SAL+ZAN
SAL+IPR
IPR

Caption to Figure S3: Comparison of antibiotic maximal efficacies (E_{max}) expressed as percentages reduction in resorufin fluorescence (viability; left panels) or crystal violet absorbance (biomass; right panels) as compared to controls (no antibiotic added) for 2-days and 11-days old naive and induced biofilms of strain R6. Top panels: moxifloxacin; bottom panels, solithromycin. Values were calculated as means ± SD of 2-8 independent experiments performed in quadruplicates (when not visible, the bars are smaller than the size of the symbols), using the Hill equation of the concentration-response curves; see also Table S4 in this Supplementary Material for numerical values). Statistical analyses: one-way ANOVA with Tukey post-test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Small letters: comparison between different types of biofilms for each growth medium; caps letters: comparison between the different growth media for each type of biofilm.

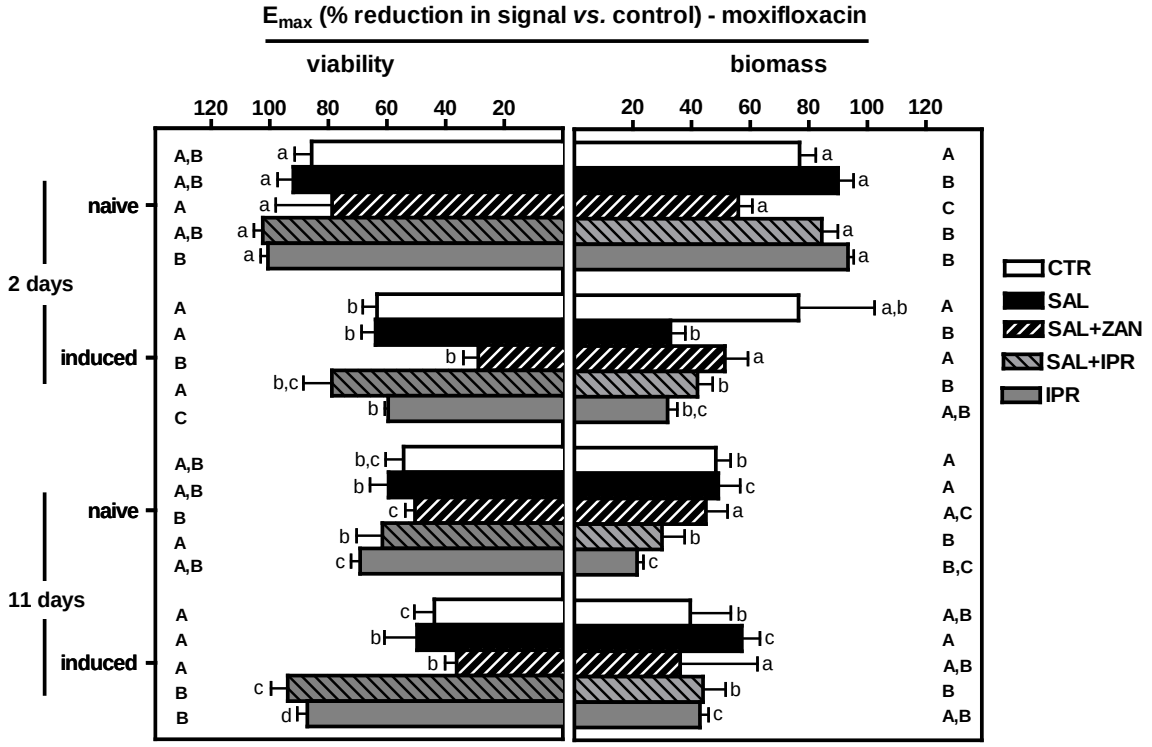
Figure S4

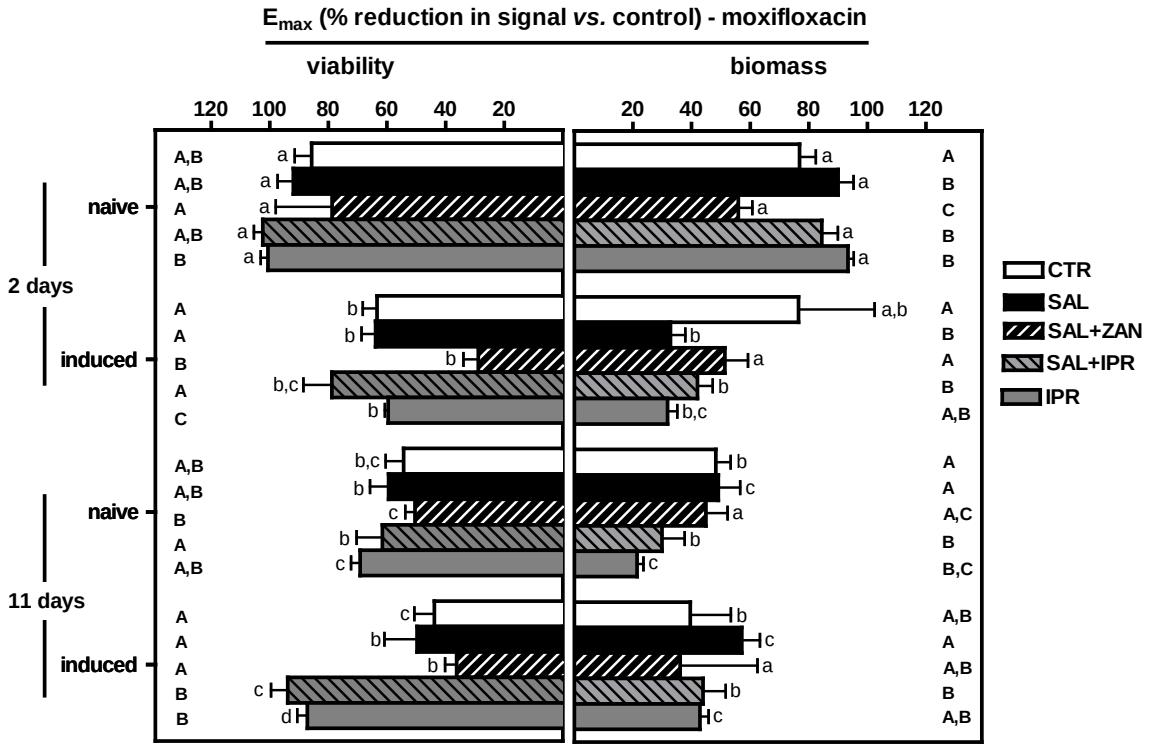


Caption to Figure S4: Comparison of antibiotic maximal relative potencies (C_{50}) expressed in X MIC towards viability (left panels) or biomass (right panels) for 2-days and 11-days old naive (n) and induced (i) biofilms of strain R6. Top panels: moxifloxacin; bottom panels,

solithromycin. Biofilms were grown Values were calculated as means ± SD of 2-8 independent experiments performed in quadruplicates (when not visible, the bars are smaller than the size of the symbols), using the Hill equation of the concentration-response curves; see also Table S4 in this Supplementary Material for numerical values). Statistical analyses: one-way ANOVA with Tukey post-test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Small letters: comparison between different types of biofilms for each growth medium; caps letters: comparison between the different growth media for each type of biofilm.

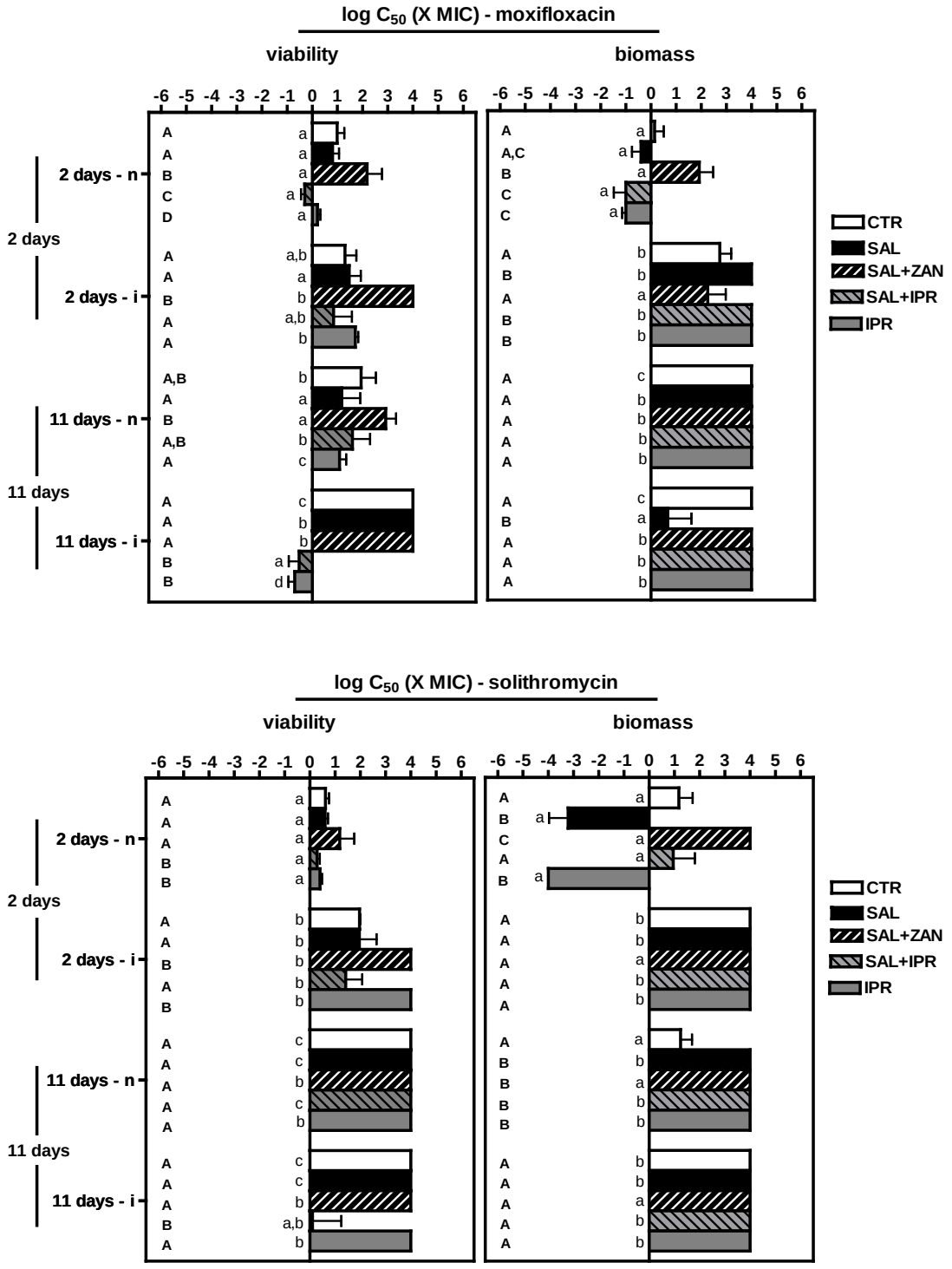
Figure S5


E$_{max}$ (% reduction in signal *vs.* control) - moxifloxacin
viability
biomass
120 100 80 60 40 20
20 40 60 80 100 120
2 days
11 days
naive
induced
CTR
SAL
SAL+ZAN
SAL+IPR
IPR


E$_{max}$ (% reduction in signal *vs.* control) - moxifloxacin
viability
biomass
120 100 80 60 40 20
20 40 60 80 100 120
2 days
11 days
naive
induced
CTR
SAL
SAL+ZAN
SAL+IPR
IPR

Caption to Figure S5: Comparison of antibiotic maximal efficacies (E_{max}) expressed as percentages reduction in resorufin fluorescence (viability; left panels) or crystal violet absorbance (biomass; right panels) as compared to controls (no antibiotic added) for 2-days and 11-days old naive and induced biofilms of strain N6. Top panels: moxifloxacin; bottom panels, solithromycin. Values were calculated as means ± SD of 2-8 independent experiments performed in quadruplicates (when not visible, the bars are smaller than the size of the symbols), using the Hill equation of the concentration-response curves; see also Table S5 in this Supplementary Material for numerical values). Statistical analyses: one-way ANOVA with Tukey post-test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Small letters: comparison between different types of biofilms for each growth medium; caps letters: comparison between the different growth media for each type of biofilm.

Figure S6



Caption to Figure S6: Comparison of antibiotic maximal relative potencies (C_{50}) expressed in X MIC towards viability (left panels) or biomass (right panels) for 2-days and 11-days old naive (n) and induced (i) biofilms of strain N6. Top panels: moxifloxacin; bottom panels,

solithromycin. Biofilms were grown Values were calculated as means ± SD of 2-8 independent experiments performed in quadruplicates (when not visible, the bars are smaller than the size of the symbols), using the Hill equation of the concentration-response curves; see also Table S5 in this supplementary Material for numerical values). Statistical analyses: one-way ANOVA with Tukey post-test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Small letters: comparison between different types of biofilms for each growth medium; caps letters: comparison between the different growth media for each type of biofilm.