Université catholique de Louvain
Medical Sector

LOUVAIN DRUG RESEARCH INSTITUTE

SUPERVISOR : Professor Françoise VAN BAMBEKE

Je suis profondément attachée au site universitaire de l'UCL-en-Woluwé pour différentes raisons : notamment, pour être née aux Cliniques universitaires St Luc le 09 novembre 1986, pour y avoir fait mes études de Pharmacie et, finalement, cette thèse de Doctorat.

Avant de débuter mes études scientifiques, mes passions étaient les Langues étrangères, la Nature et l'Histoire de l'Art, avec un goût prononcé pour l'Archéologie, la Peinture et la Musique. Mes parents et mes Professeurs m'ont enseigné le métier de Pharmacien et, de manière plus générale, celui de Professionnel de la Santé. Cette thèse aura permis de faire le lien entre ces deux parties de ma vie.

La Recherche scientifique est en effet le meilleur lieu, selon moi, pour mettre à profit son imagination au service de la Science. **Je suis heureuse d'avoir pu découvrir ce milieu extraordinaire.**

Résumé: *La **B**roncho-**p**neumopathie **C**hronique **O**bstructive (**BPCO**) est l'une des causes majeures de mortalité et morbidité au niveau mondial et les fréquentes exacerbations bactériennes constituent un facteur de risque prépondérant d'aggravation de la maladie. Le S. pneumoniae est l'un des pathogènes les plus fréquemment impliqués dans ces infections.*

Dans ce contexte, les objectifs de cette thèse étaient d'étudier les facteurs pouvant favoriser la chronicité des infections pneumococciques chez les patients bronchiteux chroniques. En collaboration avec les médecins et microbiologistes de cinq hôpitaux belges, nous avons donc collecté des isolats cliniques de pneumocoques provenant de patients BPCO ainsi que le dossier médical global de ces derniers. Ensuite, ces souches cliniques furent utiliséees afin de déterminer leur sensibilité aux antibiotiques et ainsi, évaluer le niveau de résistance en Belgique. D'autres paramètres bactériens, tels que le sérotype ou la propension à former du biofilm, et les caractéristiques physiologiques et pharmacologiques des patients furent investigués en parallèle à la résistance aux antibiotiques et en inter-connexion. Le centre d'intérêt majeur de nos études fut l'étude de la croissance de souches de référence et d'isolats cliniques de Pneumocoque au sein de biofilm. En effet, ce mode de vie bactérien particulier est reconnu comme étant impliqué dans la récurrence des infections. Dans ce contexte, nous avons développé des modèles in vitro de biofilms et les avons utilisés pour réaliser des études pharmacodynamiques d'activité antibiotique (études dose-effet) et pour déterminer quelles molécules sont les plus actives vis-à-vis des cellules pneumococciques vivant au sein d'une matrice biofilmidique. Ensuite, nous avons étudié comment les co-medications non-antibiotiques, fréquemment prescrites aux patients, peuvent moduler la production et la cohésion matricielle et ainsi, l'activité antibiotique vis-à-vis des cellules pneumococciques sessiles. En conclusion, cette thèse permet de mieux comprendre les causes éventuelles d'échec thérapeutique survenant chez les patients BPCO et de déterminer quels sont les choix d'antibiothérapie les plus adaptés pour traiter ce type d'infections. De plus, elle ouvre la voie à de nouvelles idées visant à améliorer la prise en charge des patients, en utilisant pour ce faire le principe de synergie entre co-médications et antibiotiques.

***Summary :** **C**hronic **O**bstructive **P**ulmonary **D**isease (**COPD**) is one of the most important causes of mortality and morbidity worldwide and frequent bacterial exacerbations are predominant risk factors implicated in this process. S.pneumoniae is one of the most prevalent pathogens implicated in these episodes.*

In that context, the objectives of this thesis were to investigate the parameters that can favor the chronicity of pneumococcal infections in patients suffering from chronic bronchitis. To this aim, in collaboration with physicians and microbiologists of five Belgian hospitals, we first collected pneumococcal clinical isolates coming from COPD patients and the whole patient's medical data. Secondly, we used the clinical strains to determine their susceptibility profile to antibiotics and study the prevalence of pneumococcal resistance in Belgium. In parallel, other bacterial characteristics such as strains' serotype or their ability to produce biofilms were investigated and brought into relationship with each other, with patients' medications or with the degree of pulmonary obstruction. Pneumococcal growth within biofilms was deeply investigated in this work using reference strains and clinical isolates. Indeed, this special bacterial life mode is known to be closely associated with the persistence of infections. In this context, we set up in vitro biofilm models and used them to perform pharmacodynamic studies of antibiotic activity (dose-effect relationships) and to determine which molecules are the most active against pneumococcal cells protected by the biofilm matrix. Additionaly, we also tried to better understand how non-antibiotic drugs, frequently prescribed to the patients, can modulate the pneumococcal matrix production and cohesion and, therefore, the antibiotic activity towards sessile cells. In conclusion, this thesis helps to explore potential causes of treatment failure occurring in COPD patients, to define the most rational antibiotics choices to treat these infections and offers new ideas and perspectives to improve patients' management through the synergy of co-medications and antibiotics.

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Vandevelde Nathalie M.

Recurrence of *S. pneumoniae* infections in a context of COPD

UCL Université catholique de Louvain

LDRI LOUVAIN DRUG RESEARCH INSTITUTE

Streptococcus pneumoniae infections in a context of Chronic Obstructive Pulmonary Disease (COPD)

Study of the factors contributing to the recurrence of the disease.

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Pharm.D

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I am the Master of my Fate:
I am the Captain of my Soul.

- William Ernest Henley -

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J'ai eu la chance de pouvoir bénéficier de l'aide et du soutien d'un nombre très important de gens tout au long de ma jeune vie et serais dès lors totalement incapable de dédier ce travail uniquement à une seule personne. Je le dédie donc, avec ma plus grande affection, à chacun d'entre vous. Merci pour tout !!

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Abbreviation list

ABC : ATP Binding Cassette

AECB: Acute Exacerbations of Chronic Bronchitis

AI: Autoinducer

AHL: Acetyl Homoserine Lactone

AMPc: Cyclic Adenosine Monophosphate

ATP: Adenine triphosphate

ATS: American Thoracic Society

caMHB : cation-adjusted Mueller Hinton broth

CAP : Community-Acquired Pneumonia

CAT: COPD Assessment Test

Cbp : Choline binding protein

CCQ: Clinical COPD questionnaire

CFU: Colony-Forming Unit

CLSI: Clinical and Laboratory Standards Institute

COPD: Chronic Obstructive Pulmonary Disease

CSP: Competence Stimulating Peptide

CV: Crystal Violet

D-Ala : D-Alanine

EPS: Extracellular polymeric substance

ERS: European Respiratory Society

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FEV1: Forced Expiratory Volume in 1 second

Gal : Galactose

Glc : Glucose

GOLD: Global initiative for Chronic Obstructive Lung Disease

IL: Interleukine

Ig : Immunoglobulin

LHB: Lysed Horse Blood

Lyt : Autolysin

LVRS: Lung Volume-Reduction Surgery

MATE : Multidrug And Toxic coumpounds Extrusion

mMRC: modified Medical Research Council

MFS : Major Facilitator Superfamily

MIC: Minimal Inhibitory Concentration

Nan : Neuraminidase

Neu5Ac: N-acetylneuraminic acid = sialic acid

NOX : NADH oxidase

PBP : Penicillin-Binding Protein

PLY: Pneumolysin

Psp: Pneumococcal surface protein

PsrP : Pneumococcal serine-rich Protein

QS: Quorum-sensing

RF: Resorufin

ROS: Reactive Oxygen Species

RT-PCR : Reverse Transcription Polymerase Chain Reaction

SG : Serogroup

SGRQ: St George's Respiratory Questionnaire

ST : Serotype

TLR: Toll-Like Receptor

VC: Vital Capacity

WGA : Wheat Germ Agglutinin

WHO: World Health Organization

Introduction

1. Chronic Obstructive Pulmonary Disease (COPD)

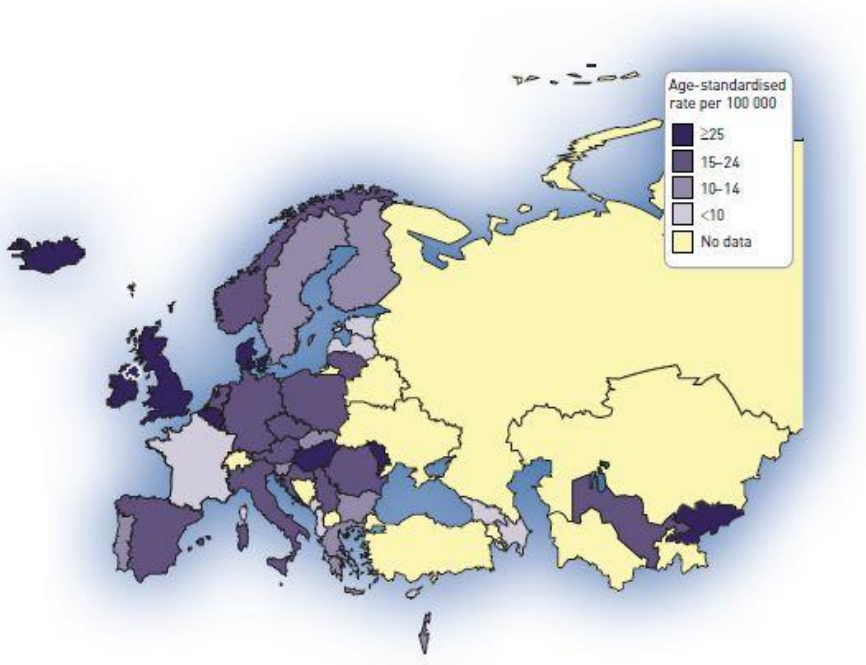
1.1. Definition and epidemiology

At the time of writing the first lines of this work, I'm turning my head in direction of the window and look to the seventh floor of the Brussels St Luc Hospital, located in front of our laboratory and occupied by the Pneumology Department. The ward tells thirty beds. More than the half of them is occupied by hospitalized patients suffering from severe Chronic Obstructive Pulmonary Disease (COPD), a non-reversible respiratory disorder, also more simply called Chronic Bronchitis.

In 2004, the World Health Organization estimated that 64 millions of persons suffered from COPD worldwide with 11 million of them living in the United States and predicted that the incidence of the disease will increase until 2030 (World Health Organization (WHO), 2013). Indeed, actual data available for the American population indicates that, nowadays, 32 millions of people are suffering from the disease.

In the 1990s, COPD, as the 6th cause of death worldwide, caused 2 million of death worldwide per year (Murray and Lopez, 1997 ; Lundback *et al.*, 2003) and was predicted to reach the third place in 2020 with 4-5 millions of death, just after ischemia and cardio-vascular diseases (Murray and Lopez, 1997; Lundback *et al.*, 2003). Recent demographic seizures showed that COPD had already reached the top 3 of mortality causes in 2010 (Minino and Murphy, 2012).

Figure 1



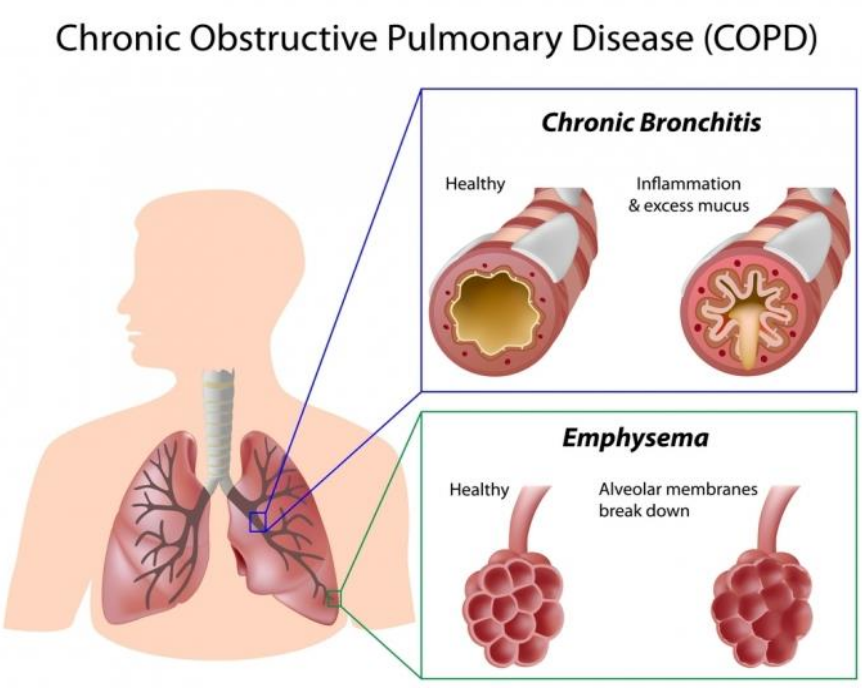
Mortality rate for chronic obstructive Pulmonary disease (COPD) (Gibson *et al.*, 2013). Data from World Health Organization World and Europe Mortality Databases, November 2011 update. Data for some countries are missing because mortality data for asthma and COPD are not reported separately.

1.2. Diagnostic

COPD is respiratory illness defined by an obstructive syndrome combining respiratory symptoms induced and explained by a chronic inflammation of the airways that is, in most of cases, related to patient's history or life mode.

Dyspnea, chronic cough and sputum production are respiratory symptoms always present in COPD patients and are mainly caused by present or previous longtime exposure to noxious substances such as tobacco or industrial dust. Fifty percents of longtime smokers are otherwise considered as future COPD patients (Lundback *et al.*, 2003) and, for approximately 40% of them, the smoking habit is maintained after the diagnostic of COPD (Anthonisen, 2007). These substances are inducing dryness of the airways accompanied by the destruction of the lung parenchyma, called emphysema (Benjamin M. Smith, 2014). In this situation, opening of small airways is diminished. This process is accompanied by a chronic inflammatory response during which the organism develops the three defense mechanisms mentioned previously, dyspnea, chronic cough and sputum production (dos *et al.*, 2012).

Figure 2

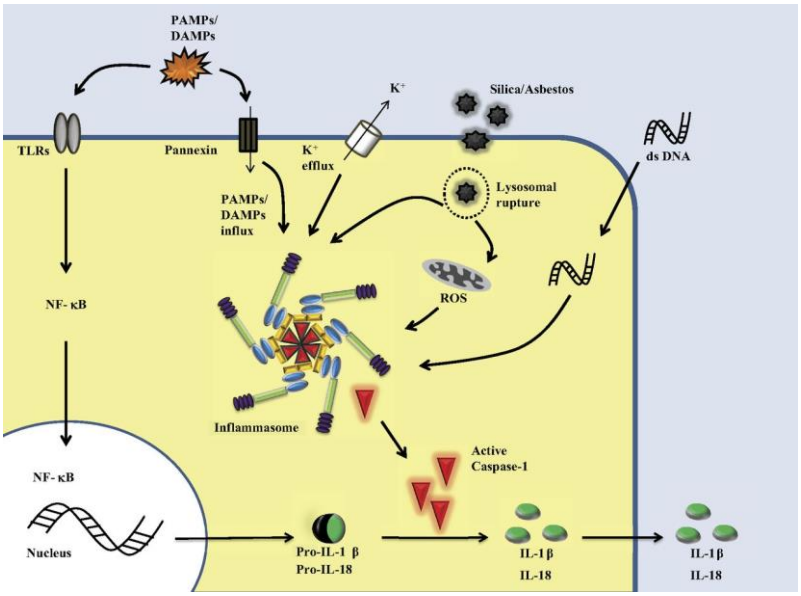


http://www.earthtimes.org/health/tai-chi-therapy-copd/2122/

The inflammation process

The inducible NLPR3 (nucleotide oligomerization domain (NOD)-like receptor containing a pyrin domain) inflammasome expressed in macrophages is actually considered to be implicated in the inflammatory response that occurs during respiratory infections, such as pneumococcal community acquired pneumonia or acute exacerbations of chronic bronchitis (dos Santos G. *et al.*, 2012). Its activation involves two main steps. Firstly, bacterial Toll-like receptors (TLR) stimulation leads to transcription of pro-IL-1β and pro-IL-18 and expression of NLRP3 secondly followed by the assembly of the NLRP3 inflammasome. It seems that in cases of pneumococcal infections, this second step is activated though the polysaccharide capsule, the major virulence factor in pneumococci (Domenech *et al.*, 2013a) and pneumolysin (PLY), a pore-forming toxin inducing K^+ release through membrane disruption (dos Santos G. *et al.*, 2012). This release, which can also be mediated by adenosine triphosphate (ATP), another activator of the inflammasome upregulated in airways of COPD, seems to be a key element of NLPR3 assembly and activation, in addition to lysosomal rupture and production of reactive oxygen species (ROS). The assembly of NLRP3 leads to activation of caspase-1 and consequently of mature IL-1 β and IL-18 released in the extracellular compartment and inducing the inflammatory response (Blasi, 2009; dos Santos G. *et al.*, 2012).

Figure 3



Activation of the inflammasome
(dos Santos G. *et al.*, 2012)

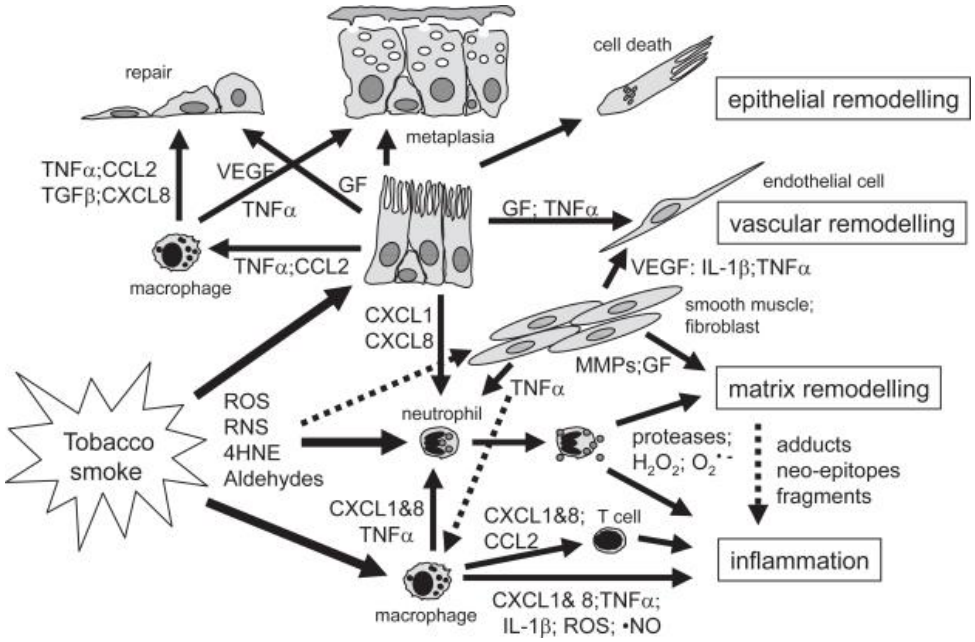
Maturation and secretion of IL-1β requires 2 signals: the "priming signal" leads to synthesis of pro-IL-1β, pro-IL-18, and other components of the inflammasome, such as NLRP3, and the second signal results in assembly of the inflammasome, activation of caspase-1, and release of mature cytokines IL-1β and IL-18 into the extracellular milieu. Currently, the nature of the second signal is debated. The 3 proposed models of activation are shown: *1*) extracellular ATP, which activates the purinergic $P2X_7$ receptor and causes subsequent recruitment of pannexin-1 hemichannel to the plasma membrane and K^+ efflux; *2*) lysosomal rupture after engulfment of crystalline or particulate agonists; and *3*) reactive oxygen species (ROS), which upregulate NLRP3 expression and activate the inflammasome. PAMP, pathogen-associated molecular pattern; DAMP, danger-associated molecular pattern; TLR, Toll-like receptor; dsDNA, double-stranded DNA.

In absence of infections, the chronic airways inflammation occurring in COPD patients is mainly attributed to their smoking habits and toxic components contained in cigarettes. In 2009, Churg and colleagues published some data showing that tobacco smoke is able to increase the levels of caspase-1 *in vivo*, favoring inflammation in absence of respiratory infections (Churg *et al.*, 2009). This is only

one example of the different pathways stimulated by tobacco components and leading to emphysema, mucus hypersecretion and chronic inflammation. These processes are dependent on different signaling cascades and require some remodeling of the architecture and integrity of epithelial, endothelial and connective tissues cells but also of the function of immune system cells. These phenomena are described here below in more detail and illustrated in Figure 4.

Approximately 5000 compounds constitute tobacco smoke and 10^7 free radicals are delivered to the smokers'airways with each puff (de Boer *et al.*, 2007). Many of them are able to induce toxic reactive compounds (*e.g.* reactive oxygen or nitrogen species [ROS, RNS], 4-hydroxy-2-nonenal [4-HNE], aldehydes) which may be responsible for (i) the oxidation of proteins, DNA and lipids, leading to direct lung injury and (ii) the induction of immune cellular responses mediated by cytokines (de Boer *et al.*, 2007). These mechanisms lead to epithelial, vascular and matrix remodeling but also to the generation of secondary metabolic reactive species keeping the inflammatory moving on (Repine *et al.*, 1997; de Boer *et al.*, 2007).

Figure 4



Simplified summary of inflammatory and remodeling mechanisms in the airways in COPD (de Boer *et al.*, 2007)

Exposure to cigarette smoke in susceptible individuals leads to an abnormal inflammation and tissue remodeling. This appears to be self-perpetuating and may be linked to infection. Tobacco smoke activates different cell types including macrophages, epithelial and smooth muscle cells to produce cytokines, growth factors or proteases. Reactive molecules in tobacco smoke stimulate airway macrophages to produce cytokines and reactive oxygen or nitrogen species. Activated macrophages and epithelial cells attract and activate inflammatory cells including monocytes, macrophages, neutrophils and T cells. Alternatively, reactive species may react with extracellular matrix (ECM), and lipid moieties causing cell damage, gene expression or oxidative stress in different cell types. Chemokines like CXCL-8 and CXCL-1 cause T cell and neutrophil chemotaxis and activation of neutrophils to degranulate proteases like elastase and MMPs, and produce reactive oxygen species like hydrogen peroxide or O2 •–. Radicals may activate proteases that in turn fragment ECM molecules and/or form ECM neo-epitopes. Oxygen radicals may also react with ECM leading to adducts or neo-epitopes. Altered or fragmented ECM molecules may stimulate inflammation and auto-immune-like reactions. Tobacco smoke may also activate smooth muscle cells and fibroblasts to produce pro-inflammatory cytokines and growth factors (GF) like VEGF, leading to Th1-mediated inflammation and vascular remodelling. Loss of epithelial cells due to direct toxicity of smoke, TNFα-induced apoptosis, or degradation of ECM, induces a repair process. Growth factors like EGF, FGF, TGFβ1 and VEGF stimulate tissue repair and vascular remodelling seen in COPD. Epithelial remodelling (squamous or mucous metaplasia, hyperplasia) may be due to excessive growth factor production or by TNFα resulting in a loss of lung clearance function and mucus hyperproduction. A-HNE, 4-hydroxy-2-nonenal; ROS, reactive oxygen species; RNS, reactive nitrogen species.

Quantification of the obstructive syndrome and COPD classification

A first categorization of COPD patients according to their respiratory symptoms, illustrated in Table 1, was made by Anthonisen and published in 1987 (Anthonisen *et al.*, 1987).

Table 1. Stratification of patients according to Anthonisen's criteria (Anthonisen *et al.*, 1987)

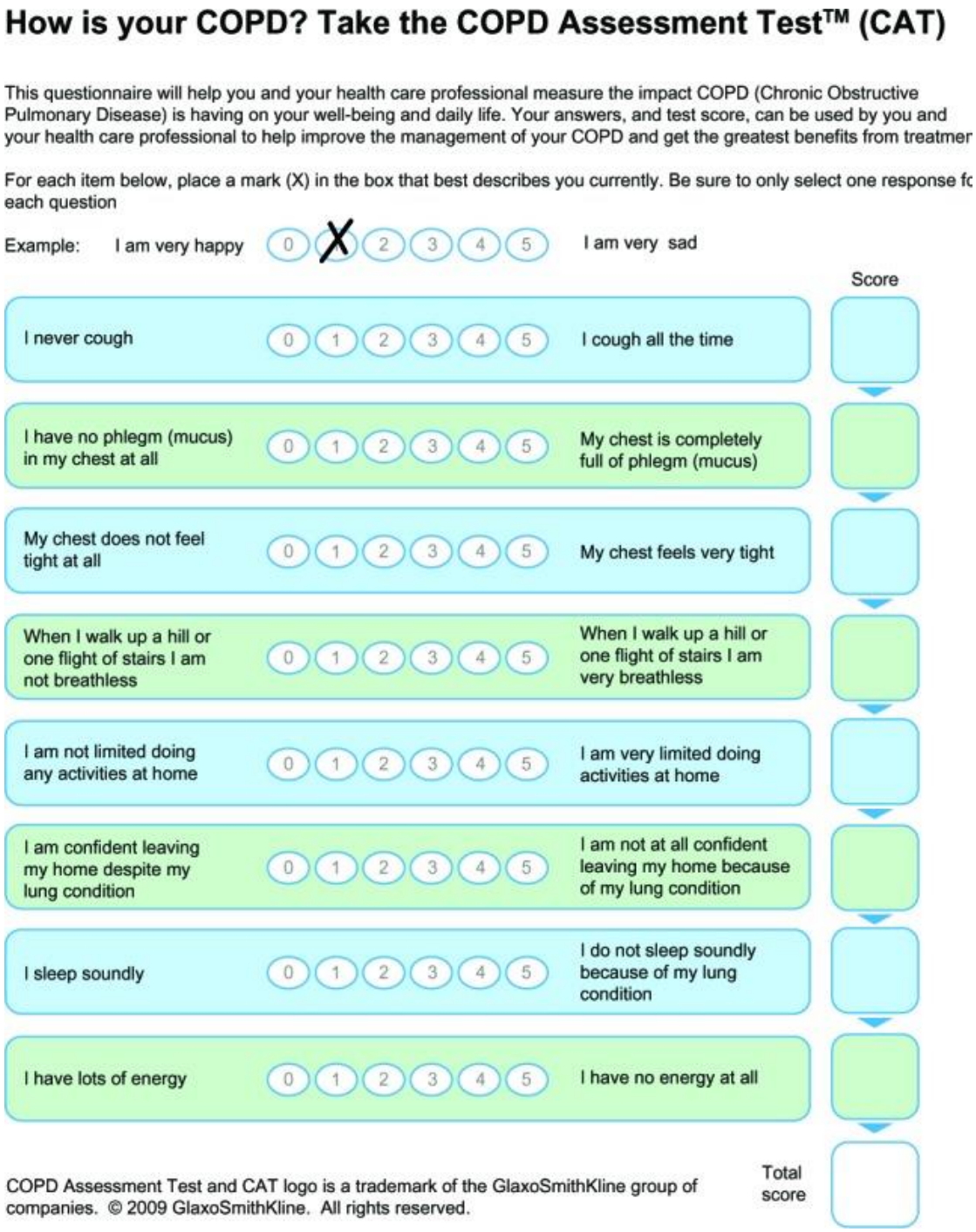
COPD Symptoms	Anthonisen classification	Stage
Dyspnea	**Severe COPD** Increase of the 3 COPD-symptoms	I
Purulent sputum	**Moderate COPD** 2 of the 3 COPD-symptoms are increased	II
Increase of sputum production	**Mild COPD** Increase of only 1 COPD-symptom with one of the following symptoms: - Increase of the cough - increase of the sibilant rale - Increase of 20% of the respiratory or heart rate - undiagnosed fever - a lower respiratory tract infection in the last 5 days	III

Further patients classification was performed using (i) qualitative evaluation of changes in the patient's quality of life because of these respiratory symptoms and (ii) quantitative spirometric measurements of the respiratory function. These two types of characterization are grouped into the "Global Initiative for Chronic Obstructive Lung Disease (GOLD)" classification (GOLD, 2014) and described hereafter.

Because the severity of respiratory symptoms described in Table 1 is difficult to evaluate in clinical practice and affect patient's quality of life, numerous questionnaires have been developed to evaluate and take other symptoms of the everyday life into account (van der Molen T. *et al.*, 2013). The goal is to give to physicians a more complete understanding of the disease impact on their patients, not only regarding clinical status of the airways but also activity limitation and emotional dysfunction (van der Molen T. *et al.*, 2013). The most frequent used and known are the modified Medical Research Council (mMRC), the COPD assessment Test (CAT, see Figure 5), the clinical COPD questionnaire (CCQ), and the St George's Respiratory Questionnaire (SGRQ) (van der Molen T. *et al.*, 2013). The two first mentioned are now included in GOLD classification, translating the importance of a multidimensional assessment and management approach (GOLD, 2014). The mMRC dyspnea scale is used for grading the effect of breathlessness on daily activities. The CAT, developed in 2009, investigates 8 items including respiratory symptoms, physical activity and capacity, quality of sleep and patients own feelings and confidence (e.g. ability to live at home). The CCQ, developed in 2003, assesses 3 domains: symptoms, functional state, and mental state with a score varying between 0 and 6 (least to worst impairment). Finally, the St George's Respiratory Questionnaire, designed in 1991 is the more

1991 is the more complete with 50 items investigated (Jones *et al.*, 1991). However, a good correlation was found between the CAT, CCQ, and St George's Respiratory Questionnaire (van der Molen T. *et al.*, 2013).

Figure 5



How is your COPD? Take the COPD Assessment Test™ (CAT)

This questionnaire will help you and your health care professional measure the impact COPD (Chronic Obstructive Pulmonary Disease) is having on your well-being and daily life. Your answers, and test score, can be used by you and your health care professional to help improve the management of your COPD and get the greatest benefits from treatmer

For each item below, place a mark (X) in the box that best describes you currently. Be sure to only select one response fc each question

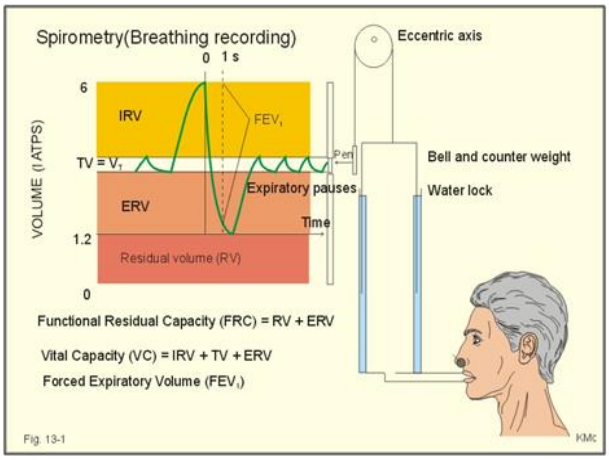
Example:	I am very happy	0 **X** 2 3 4 5	I am very sad
			Score
I never cough	0 1 2 3 4 5	I cough all the time	
I have no phlegm (mucus) in my chest at all	0 1 2 3 4 5	My chest is completely full of phlegm (mucus)	
My chest does not feel tight at all	0 1 2 3 4 5	My chest feels very tight	
When I walk up a hill or one flight of stairs I am not breathless	0 1 2 3 4 5	When I walk up a hill or one flight of stairs I am very breathless	
I am not limited doing any activities at home	0 1 2 3 4 5	I am very limited doing activities at home	
I am confident leaving my home despite my lung condition	0 1 2 3 4 5	I am not at all confident leaving my home because of my lung condition	
I sleep soundly	0 1 2 3 4 5	I do not sleep soundly because of my lung condition	
I have lots of energy	0 1 2 3 4 5	I have no energy at all	
			Total score

Illustration of the COPD assessment Test (CAT) (van der Molen T. *et al.*, 2013)

Concerning quantitative measures, spirometry is used to quantify the level of patient's airflow limitation over years (see figure 6).

This method involves measuring two respiratory parameters: (i) the Forced expiratory volume in 1 second (FEV1) representing the volume of air forcibly blown out during one second of forced exhalation, after complete inhalation and (ii), the Vital capacity (VC) representing the total amount of air forcibly exhaled after a complete inhalation. The GOLD guidelines for diagnosis and management of COPD state that airflow obstruction is present when the FEV1/FVC ratio is less than 70 when measured after administration of a short-acting inhaled bronchodilator (GOLD, 2014). Based on spirometric measures, the scores of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) allow classifying COPD severity in four stages, from the least (1) to the more severe (4). However, using only this fixed cutoff of airflow limitation, there would be an overdiagnosis of COPD (GOLD, 2014).

Figure 6



The residual volume (RV) represents the volume of air left in the lungs after a maximal expiration. The vital capacity (VC) is the maximum volume of air that can be exhaled after a maximal inspiration. The inspiratory reserve volume (IRV) is the quantity of air that can be inhaled from a normal end inspiration position. The tidal volume (V_T) is the volume of air inspired and expired with each breath. The expiratory reserve volume (ERV) is the amount of air that can be exhaled from the lungs from a normal end-tidal expiratory position that is characterized by a relaxed expiratory pause. http://www.zuniv.net/physiology/book/chapter13.html

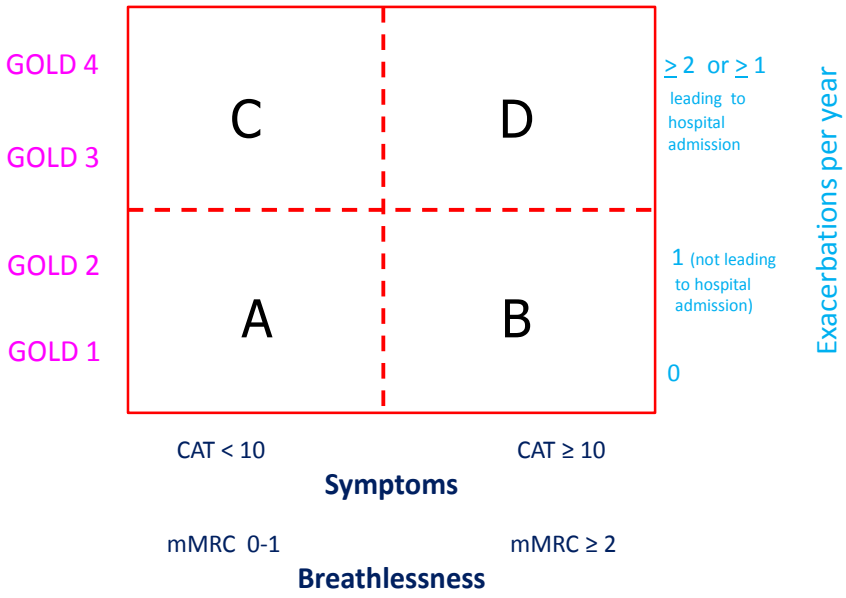
Table 2

Stage	Spirometric FEV1 measures
I	**Mild COPD**: FEV1 ≥ 80% predicted
II	**Moderate COPD**: 50% ≤ FEV1 < 80% predicted
III	**Severe COPD:** 30% < FEV1< 50% predicted
IV	**Very severe COPD:** FEV1 < 30% predicted

Stratification according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD, 2014)

Taking the frequency of exacerbations per year, patients quality of life and spirometric classification into account, the Global Initiative for Chronic Obstructive Lung Disease (GOLD, 2014) classifies patients in four categories (see Figure 7 and Table 3 here below) and recommends the type of respiratory treatment that should be administrated.

Figure 7



Schematic representation of the COPD patients classification according to GOLD criteria (GOLD, 2014)

Table 3. Classification of COPD patients according to the actual GOLD criteria (GOLD, 2014)

Patient category	Characteristics	Spirometric classification	Exacerbations per year	Score at the COPD Assessment Test (CAT)	Score at the Modified Medical Research Council Questionnaire (mMRC)
A	Low risk, less symptoms	GOLD 1-2	≤ 1	< 10	0-1
B	Low risk, more symptoms	GOLD 1-2	≤ 1	≥ 10	≥ 2
C	High risk, less symptoms	GOLD 3-4	≥ 2	< 10	0-1
D	High risk, more symptoms	GOLD 3-4	≥ 2	≥ 10	≥ 2

1.3. Pharmacologic management of respiratory symptoms

Concerning precisely drugs treatments, COPD patients are receiving several types of medications to treat their respiratory symptoms. These are short or/and long-acting bronchodilators, associated or not with inhaled corticosteroids to deal with the obstructive syndrome. Other molecules, such as theophilline or carbocysteine, a mucolytic agent, may also complete patient's management if required. The treatment administered is defined by the patient GOLD score previously described and illustrated in Table 4.

Table 4. Initial Pharmacologic Management of COPD*

Patient Category	Recommended First Choice	Alternative Choice	Other Possible Treatments**
A	Short-acting anticholinergic ***or*** Short-acting β_2-agonist	Long-acting anticholinergic ***or*** Long-acting β_2-agonist ***or*** Short-acting anticholinergic + S hort-acting β_2-agonist	Theophylline
B	Long-acting anticholinergic ***or*** Long-acting β_2-agonist	Long-acting anticholinergic + Long-acting β_2-agonist	Short-acting β_2-agonist ***and/or*** Short-acting anticholinergic Theophylline
C	Inhaled corticosteroids + Long-acting β_2-agonist ***or*** Long-acting anticholinergic	Long-acting anticholinergic + Long-acting β_2-agonist ***or*** Long-acting anticholinergic + phosphodiesterase-4 inhibitor ***or*** Long-acting β_2-agonist + phosphodiesterase-4 inhibitor	Short-acting β_2-agonist ***and/or*** Short-acting anticholinergic Theophylline
D	Inhaled corticosteroids +Long-acting β_2-agonist ***and/or*** Long-acting anticholinergic	Inhaled corticosteroids + Long-acting β_2-agonist + Long-acting anticholinergic ***or*** Inhaled corticosteroids + Long-acting β_2-agonist + phosphodiesterase-4 inhibitor ***or*** Long-acting anticholinergic + Long-acting β_2-agonist ***or*** Long-acting anticholinergic + phosphodiesterase-4 inhibitor	Carbocysteine Short-acting β_2-agonist ***and/or*** Short-acting anticholinergic Theophylline

* : Medications in each box are mentioned in alphabetical order, and therefore not necessarily in order of preference
** : Medications in this column can be used alone or in combination with other options in the Recommended First Choice and Alternative Choice columns

Global Initiative for Chronic Obstructive Lung Disease (GOLD) - GOLD, 2014

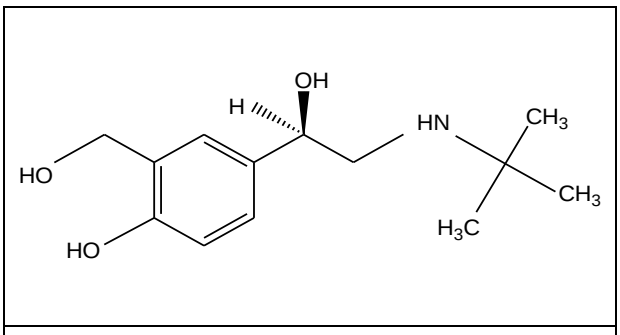
β_2-agonists bronchodilators

These drugs are binding to β2-adrenergic receptors of smooth muscles, increasing AMPc through the activation of an adenylyl cyclase. Increased levels of AMPc are activating the AMPc-dependent protein kinase A that induces relaxation of airways smooth muscles. The effect of short acting β2-agonists (salbutamol), used as crisis treatment, wears off within 4 to 6 hours when long-acting (formoterol, salmeterol, indacaterol), used as maintenance treatment, show duration of action at least equal to 12 hours.

Actually, indacaterol is considered as the most active long-acting molecules β2-agonist with 2 main advantages: reaching the peak serum concentration within 15 minutes and having a 24h-therapeutic action (Seth *et al.,* 2013; Ridolo *et al.,* 2013). Its efficacy is comparable to that of tiotropium described here below (Seth *et al.,* 2013; Ridolo *et al.,* 2013).

β2-agonists are now also recognized as having anti-inflammatory properties by modulating the activity of immune system cells (monocytes/macrophages, basophils, eosinophils, mast cells, neutrophils, T lymphocytes) or directly reducing inflammation in cells of respiratory tissues (epithelial cells, fibroblasts, smooth muscle cells) through AMPc-dependent or AMPc-independent mechanisms, with long-acting molecules, especially formoterol, being the most effective (Theron et al, Drug Design, Development and Therapy 2013:7 1387–1398). Figure 8 shows the structure of salbutamol, used as exemplative β2-agonist molecule in this work.

Figure 8



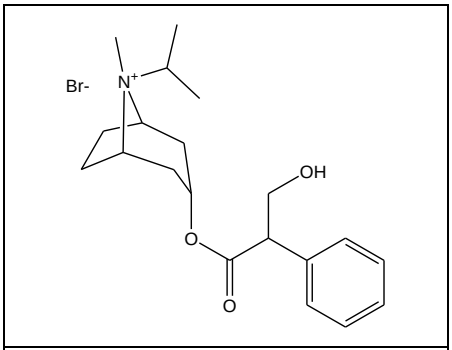
salbutamol

Anticholinergic bronchodilators

These drugs are antagonizing acetylcholine bronchoconstriction effect by binding and blocking its muscarinic (M) receptors. Short-acting anticholinergics (ipratropium bromide) are binding to receptors M2 and M3 offering a bronchodilation during 8 and more hours (GOLD, 2014) and long-acting (tiotropium bromide and glycopyrronium bromide) are binding receptors M1 and M3, reducing/inhibiting bronchoconstriction during more than 24 hours (GOLD, 2014).

A recent systematic review and network meta-analysis has compared efficacies of tiotropium bromide, glycopyrronium bromide and aclidinium bromide, a new long-acting anticholinergic, not yet available on the Belgian market but already approved in other European countries and in the USA as a maintenance treatment in adults suffering from COPD. The main outcomes of interest were the Forced Expiratory Volume in 1 second (FEV_1) at 12 weeks and 24 weeks and St George's Respiratory Questionnaire (SGRQ) total score at 12 weeks and 24 weeks. The main conclusions are that, based on reporting efficacy outcome in terms of bronchodilator (FEV1), aclidinium 400 µg bromide twice a day is expected to be comparable to tiotropium 5 µg once a day, and glycopyrronium 50 µg once a day at 12 and 24 weeks. However, compared to tiotropium 5 µg, at 24 weeks, the drug is expected to offer a best efficacy according to the SGRQ total score (Karabis *et al.*, 2013). Figure 9 shows the structure of ipratropium bromide, used as exemplative anticholinergic bronchodilator in this work.

Figure 9



Ipratropium bromide

Inhaled corticosteroids

These drugs (for e.g. beclomethasone, fluticasone, budesonide, ciclesonide are available in Belgium) are shown and described as, through inducing a decrease of the inflammation process occurring in the airways, improving respiratory symptoms, quality of life and decreasing the risk of exacerbations (GOLD, 2014). Their mechanism of action has been studied *in vitro*: inhaled corticosteroids are binding to macrophages cytoplasmic glucocorticoid receptors. The complex formed migrates to the nucleus where there is some repression of pro-inflammatory genes with a decrease of the LPS-stimulated transcription of cytokines IL-6, TNFα and neutrophil chemoattractant CXCL8 (= IL-8) (Plumb *et al.*, 2013). However, their use remains controversial (Miravitlles, 2013; GOLD, 2014). Mainly because their anti-inflammatory properties lead to a decrease of function of the host defense cells, favoring the risk of COPD patients to develop pneumonia observed in very large clinical studies (Suissa *et al.*, 2013; Janson *et al.*, 2013). This is particularly observed for fluticasone, less frequently for budesonide, and can be explained by pharmacokinetics differences between these two compounds. Indeed, fluticasone is more potent in terms of interactions with intracellular steroids receptors, more lipophilic (and therefore penetrates more through membrane) and possess a longer half-life, increasing all its related side-effect, including pneumonia (Suissa *et al.*, 2013). However, it seems that this deleterious effect is dose-dependent (when >200mg per day) and time-dependent (especially when longer than 10 months) (Suissa *et al.*, 2013) and that the increased risk of pneumonia was not accompanied by an increased risk of pneumonia-related mortality (Sibila *et al.*, 2013).

Theophylline

This methylxanthine alkaloid possesses a bronchodilator and anti-inflammatory activity through inhibition of phosphodiesterase, increasing levels of AMPc and by inhibiting leukotrienes.

Phosphodiesterase-4 inhibitors

These drugs (e.g. roflumilast) are reducing inflammation through increase of AMPc levels. This type of drugs is actually not available on the Belgian market.

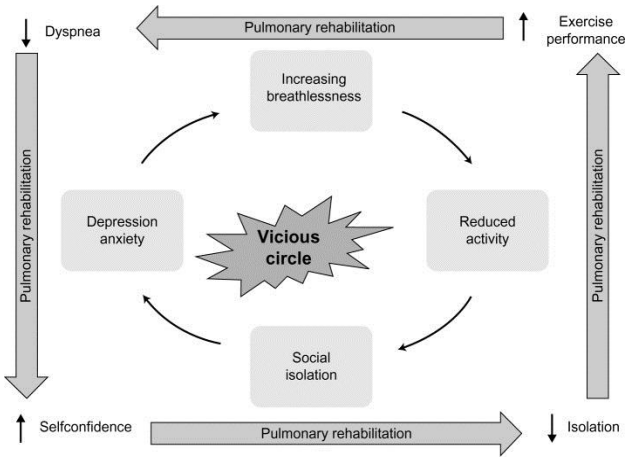
Carbocysteine

This cystein-derived mucolytic drug does not belong to the GOLD therapeutic recommendations but deals with sputum viscosity through reduction of disulfide bridges and is, therefore, sometimes used to reduce the obstructive syndrome.

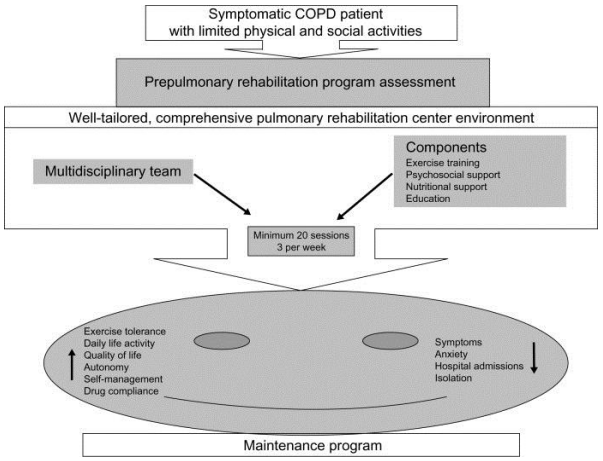
1.4. Non Pharmacologic options

In severe patients, long-term (>15 hours/day) oxygen therapy helps to decrease the risk of hospitalization and mortality, such as Lung volume-reduction surgery (LVRS) that allows to decrease hyperinflation by removing parts of the patient's lungs and lung transplantation (Gegick *et al.*, 2013; Suh *et al.*, 2013). Pulmonary rehabilitation is the most effective nonpharmacological intervention for improving the health status in COPD (Corhay *et al.*, 2014; Suh *et al.*, 2013;Troosters *et al.*, 2013) and is defined by the American Thoracic Society and the European Respiratory Society (ATS/ERS) Task Force as a comprehensive intervention based on a thorough patient assessment followed by patient-tailored therapies, which include, but are not limited to, exercise training, education and behavior change, designed to improve the physical and emotional condition of people with chronic respiratory disease and to promote the long-term adherence of health-enhancing behaviors (Spruit *et al.*, 2013). The schematic representation of Figure 10, here below, helps to understand how pulmonary rehabilitation improves the respiratory function and physiological conditions and tackles the vicious circle of symptoms leading to patient's social isolation and depression (Corhay *et al.*, 2014).

Figure 10



COPD's "vicious" circle. Corhay *et al.*, 2014.



Summary of the steps and benefits of pulmonary rehabilitation Corhay *et al.*, 2014.

1.5. Comorbidities

Comorbidities are traditionally defined as medical disorders co-existing with the primary disease of interest (Sin *et al.*, 2006). In cases of patients suffering from chronic obstructive pulmonary disease, we are mainly talking about respiratory pathologies (pneumonia and pulmonary vascular disease), cancers and cardiovascular diseases (heart failure, arrhythmia, acute myocardial infarction, hypertension, and ischemia) but also, to a lesser extent, to neurologic and psychiatric disorders (cognitive impairment, depression and anxiety), obstructive sleep apnea (OSA), diabetes, osteoporosis, cataract and glaucoma (Sin *et al.*, 2006; Soriano *et al.*, 2005; de Miguel *et al.*, 2013; Verbraecken and McNicholas, 2013; Visca *et al.*, 2013; Wells and Dransfield, 2013; Sundar *et al.*, 2014; Bratek *et al.*, 2014). It's therefore easy to understand that COPD patients, because of their respiratory symptoms and co-morbidities, are highly polymedicated. Comorbidities diminish the quality of life, translated by a high score to the Assessment Test of the St. Georges Respiratory Questionnaire (SGRQ) evaluating COPD patient's testimony (Miyazaki *et al.*, 2014) mentioned before.

When analyzing relationships between COPD and comorbidities, it would be interesting to examine which are the mechanisms involved and to consider the concept of causation with more attention: do co-pathologies increase the rate of mortality in COPD patients or does COPD increase patients risk to develop comorbidities?
It seems that comorbidities are interdependent on each other and that the mechanisms implicated are highly depend on the persistent and systemic inflammatory situation occurring in patients and already previously mentioned (Sin *et al.*, 2006).

For the relationship between COPD and malignant lesions, there is good evidence that obstructive syndrome is an important risk factor for cancer (Sin *et al.*, 2006). This could already been demonstrated more than 10 years ago through, among other studies, the first National Health and Nutrition Examination Survey (NHANES I) performed on 5,402 patients followed during 22 years (Mannino *et al.*, 2003).

Cancer in COPD patients is often attributed to severe and chronic tobacco addiction but it seems that the key parameter for developing tumors would be the chronic inflammation. Indeed, it has already been published that cytokines, such as IL-18, up-regulate pro-oncogenes and down-regulate suppressor oncogenes (e.g. p53) and thus apoptosis (O'Byrne and Dalgleish, 2001; Chiarugi *et al.*, 1998). This can explain why a clear link between COPD and lung cancer is also observed in absence of active smoking (Wasswa-Kintu *et al.*, 2005).

Considering now the link between cardio-vascular disorders and COPD, the rate of patients recorded as receiving a treatment for coronary heart disease is 2-3 times higher in cases of chronic bronchitis

than without COPD ($p<0.001$) (Berger *et al.*, 2004). However, COPD and cardiac co-pathologies are mutually playing the role of cause and effect, cardio-vascular disorder inducing higher mortality rates (approximately 4-times higher) of hospitalized COPD patients (Gulsvik *et al.*, 1978) and lung disease leading to 2-times more frequent mortality caused by cardio-vascular acute disorder (Stavem *et al.*, 2005). Once again, the systemic inflammation involved in the pathogenicity of chronic bronchitis may favor cardiac disorder through the atherosclerosis process (Sin *et al.*, 2006). Inflammatory cytokines (e.g. IL-6) and leucocytes (e.g. CD8+ T-cells) involved in the inflammation response occurring during infections of chronic bronchitis contribute to the development of pulmonary hypertension and vascular disease, also favored by cardiac and sleeping disorders (Wells and Dransfield, 2013).

Concerning cognitive impairment, it seems that causes are hypoventilation, leading to brain hypoxia and neuronal damage, and cardiac diseases that may increase the risk of cerebral hypoperfusion and microemboli (Singh *et al.*, 2013). Hypoventilation is also responsible of sleeping apnea observed in 5-10% of COPD patients (Verbraecken and McNicholas, 2013). For psychiatric symptoms, it seems that COPD frequent hospitalization and co-morbidities decreasing the quality of life are not the only explaining factors but that, once again, inflammation plays an important role. Indeed, cytokines also possess receptors in the nervous system and their brain signaling regulates several types of neurotransmissions. Therefore, increased levels of IL-6, IL-1β or TNF-α lead to cognitive impairment, depression and anxiety (Salim *et al.*, 2012; Bratek *et al.*, 2014). The psychological impairment itself favors sleeping disorders (Bratek *et al.*, 2014). In addition to this, chronic use of some drugs such as corticosteroids causes mental side effects with an impact on patient's social life.

1.6. Which place for clinical pharmacy in COPD management?

Clinical Pharmacy is a branch of Pharmaceutical Science in which pharmacists, in close collaboration with physicians and nurses, provide patient care to optimize medication (2008). In most of cases, this type of patient's management takes place in the hospital, at patient bedside, even if it's also still developing in community pharmacies (Bugnon *et al.*, 2012). The goal is to optimize drug safety (e.g. by minimizing, through medical anamnesis and patient's education, the risk of adverse effect related to drug interactions), to promote an economical drug use at patient and hospital levels and to ensure patients follow-up and adherence to treatment after hospitalization (Dalal *et al.*, 2012; Garcia-Aymerich *et al.*, 2007).

Medication adherence

An Australian team published recent data indicating that non adherence to inhaled and oral medication in COPD patients was very high (40-60%). The results were obtained through direct (e.g. measurements of drug amount in blood serum) or indirect (e.g. patients self-report) adherence records. The main reasons for this seem to be an ineffective use of inhalers (in more than 30% of cases), a complicated drug administration schedule, a patient misunderstood of the drug's mode of action, translated in 50% of cases by underuse of the maintenance treatment in absence of exacerbations and, at the opposite, drug overuse in most than 50% of cases during episodes of distress (Bryant *et al.*, 2013).

It is clear that pharmacists may play an important role in reducing these problem causes. The most important one is to optimize the dose regimen prescribed by having close discussions with physician at the hospital (Bryant *et al.*, 2013). A recent study has demonstrated that age-associated pulmonary changes, such as physical and cognitive impairment, frequently found in elderly COPD patients, favor misuse of pressurized metered dose inhalers (MDIs) and dry powder inhalers (DPIs) and poor medication adherence. Adaptation of delivery devices is therefore also crucial over the disease progression and good solution seems to change for nebulizer delivery systems (Taffet *et al.*, 2014).

Improving drug adherence by patient follow-up at home

Some pulmonary delivery systems have been set-up to favor patient adherence by recording the date and the time of each administration (e.g. Nebuliser Chronolog feedback system) (Nides *et al.*, 1993). Multi-disciplinary interventions, including e.g. nurse visit during the 3 first days after discharge also help to have a correct inhaler use. Patients follow-up by pharmacists phone-calls given 3 and 9 months after hospitalization also increased adherence to treatment and confidence (Garcia-Aymerich *et al.*, 2007). Finally, a very recent pilot study performed through an informatics follow of patients at home

(Internet-linked tablet computer-based intervention (the EDGE platform)) showed good results. The platform incorporates (i) daily patients answers to questions about their respiratory symptoms and quality of life, (ii) offers information (personalized plans for self-management, videos and articles about COPD, its treatment, including correct use of inhalers and educational information (smoking cessation ,diet and physical activity)) and (iii) possible contact with a respiratory nurse of the hospital. The data entered onto the EDGE platform is reviewed 2 times a week by a clinician to identify technical problems and review clinical data (Farmer *et al.*, 2014).

Which role for community pharmacists in adherence?

It has been demonstrated that short (3-5 minutes) counseling given by pharmacists at each delivery significantly improves adherence to drugs (Bryant *et al.*, 2013). Pharmacist may also play a role in diagnostic. Indeed, because COPD in mild and moderate stages remains highly underdiagnosed and that early diagnostic helps to prevent morbidities and further deterioration of the respiratory function, several recent programs have been set-up to improve asthma and COPD early diagnostic and treatment adherence by involving community pharmacists (Fathima *et al.*, 2013). Concerning patients suffering from chronic bronchitis, the aim was mainly to identify individuals at risk. A first research program conducted in 2010 by University of Cincinnati showed that through (i) asking to patients coming to the pharmacy to fullfill the "COPD Population screener questionnaire" and through (ii) according the possibility to pharmacist to make some spirometric measurements, they could identify individuals at risk (approximately 15% of the population investigated) and referred them to a physician (Fuller *et al.*, 2012). To perform this, special training in knowledge of respiratory diseases was given to pharmacists at the beginning of the program. Participants were recruited by their pharmacists based on their age, smoking status and respiratory symptoms. Another pilot study made in 2007 in Spanish community pharmacies and conducted in the same way (questionnaire + spirometric measures) showed similar results (Castillo *et al.*, 2009). Both studies demonstrated that community pharmacists were successful in screening patients at high risk of COPD through spirometry testing, with respectively 99% (Fuller *et al.*, 2012) and 73% (Castillo *et al.*, 2009) of COPD diagnostics confirmed by specialists at the hospital. This procedure offers significant improvements in patients follow by the pharmacist with at the end, better results in drug adherence and smoking cessation (Fathima *et al.*, 2013).

1.7. Acute Exacerbations of Chronic Bronchitis (AECB)

In addition to respiratory symptoms and chronic inflammation, 2 or 3 times per year patients develop infectious episodes of acute exacerbations (AECB) that are, with cardiovascular comorbidities, the most common cause of hospitalization and decrease of the quality of life (Anthonisen, 2002). During these episodes, all COPD respiratory symptoms are increased and if we more specifically focus on sputum, it becomes overproduced and purulent (Anthonisen, 2002). Sputum, coughed up from the lower airways, contains the mucus, a secretion produced by airways tissue linings and composed of water, polysaccharides, proteins, immunoglobulins, ions, extracellular DNA,…

Mucus naturally covers epithelial cells and protects the lung system by retaining several small particles such as dust, pollutants or allergens but also microorganisms (e.g. bacteria). During infectious episodes, this mucus can therefore play also a protective and proliferative role for pathogens by offering them a rich environment conducive to growth. This may lead to bacterial persistence in the airways and recurrence of infections such as in the case of acute exacerbations of chronic bronchitis.

Bacterial pathogens involved in acute exacerbations of chronic bronchitis

In fifty to eighty percents of infectious exacerbations of chronic bronchitis, bacterial pathogens are found in COPD patients (Eller *et al.*, 1998). These bacteria, widely described in the literature, are all belonging to species causing, among others, respiratory infections. However, the relative incidence of specific pathogens varies depending on the severity of the obstructive syndrome (Eller *et al.*, 1998; Sethi and Murphy, 2001). During mild stage, *Streptococcus pneumoniae* and *Staphylococcus aureus* and *Haemophilus influenzae* are the most frequent pathogens found in sputum samples, followed by Pseudomonas ssp, Enterobacteria, and finally, *Moraxella catarrhalis* (Eller *et al.*, 1998; Sethi and Murphy, 2001). The incidence of pneumococcal infections remains stable and high for all COPD severity stages (Eller *et al.*, 1998; Ko *et al.*, 2007), making *S. pneumoniae* one of the most important pathogens in AECB (Aydemir *et al.*, 2014). At the opposite, some pathogens, such as *P. aeruginosa*, less frequent in poorly obstructed patients, become more frequent in severe COPD patients (Eller *et al.*, 1998; Ko *et al.*, 2007; Rosell *et al.*, 2005). The chronicity of these airways infections favors chronic inflammation and therefore, also reduces patient's ability to breathe and quality of life (Sethi and Murphy, 2001).

2. *Streptococcus pneumoniae*

2.1. Definition and epidemiology

Streptococcus pneumoniae, also called Pneumococcus, is a Gram positive, alpha-hemolytic and aerotolerant bacterium for which commensally carriage in nasopharynx exists in healthy individuals, in respectively 20-40 % of cases in young children and in 5-10% of adults (Domenech *et al.*, 2009). Its outer-structure is composed of a membrane surrounded by a cell wall rich in peptidoglycans and also by a capsule forming the outermost layer (Kadioglu *et al.*, 2008).

However, in more vulnerable populations such as elderly ($\geq$ 65 years), early childhood (< 2 years) or immunocompromised patients, Pneumococcus may become pathogenic, causing local infection that may in some cases spread to deeper airways, causing infections such as otitis media, pneumonia or acute exacerbations of chronic bronchitis and in severe situations, also spread through bloodstream to other locations of the human body, causing then septicemia and/or internal organs infections, for e.g. endocarditis, osteomyelitis or meningitis (Perez-Trallero *et al.*, 2011; AlonsoDeVelasco *et al.*, 1995).

This bacterium is therefore a major cause of mortality worldwide. In 2007, the World Health Organization estimated that *S. pneumoniae* caused 1.6 million of death per year, especially in developing countries and involving children under 5 in 40 to 60% of cases (Trappetti *et al.*, 2013).

2.2. Main virulence factors (see Figure 12)

The polysaccharide capsule (200-400nm thick) is considered as the major pneumococcal virulence factor (Domenech *et al.*, 2013a), mainly because of its anti-phagocytosis protective properties (Kadioglu *et al.*, 2008). These seem to be explained by the highly charged character at physiological pH of its polysaccharides, conferring a protection by limiting interactions with phagocytes and opsonization by resistance to complement C3 deposition (Kadioglu *et al.*, 2008; Melin *et al.*, 2009).

It seems that expression of the capsule also participates to the infection process by reducing entrapment in the bronchial mucus and thereby, allowing a better access to eukaryotic cells epithelial surfaces (Kadioglu *et al.*, 2008). Fernebro *et al*. also reported in 2004 that the capsule may also provide an advantage for bacteria by affording them resistance to spontaneous or antibiotic induced autolysis. This could be demonstrated for penicillin and vancomycin (Fernebro *et al.*, 2004). Finally and at the opposite, it seems that down-regulation of the capsule may offer an advantage for *S. pneumoniae* by increasing its biofilm production (Hall-Stoodley *et al.*, 2008). However, this concept remains actually not very clear (Moscoso *et al.*, 2006). Pneumococcal growth within biofilm will be discussed further in more details.

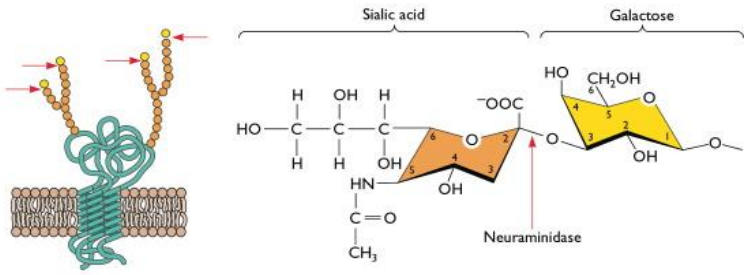
Some pneumococcal proteins may also play a role of major virulence factor. Among them may be mentioned Choline-binding protein A (CbpA or PspC), pneumococcal surface protein A (PspA), autolysin A (LytA), pneumolysin (Ply), neuraminidase A and NADH oxidase (NOX) (Kadioglu *et al.*, 2008; Muchnik *et al.*, 2013; Yu *et al.*, 2001). Because of their importance in virulence and/or implication in experiments raised in this thesis work, they are described in more details here below, in order of their implication in the infection process (from adhesion to cell death).

Pneumococcal surface protein A (PspA) is located on the cell wall of all strains of *S. pneumoniae*. The protein contains five domains (a signal peptide, a charged α-helical region of nucleotides, a proline-rich domain, a choline-binding domain and a C-terminal amino acid tail allowing its attachment to lipoteichoic and teichoic acids, two types of membrane-attached wall polysaccharides). On the basis of divergences in the nucleotide sequence of the α-helix, PspA is classified into 3 families (A1, A2 and A3) (Hotomi *et al.*, 2013; Jedrzejas, 2001). PspA confers a protective role for pneumococci against opsonization by interfering with their fixation to complement component C3 and against lactoferrin by its lactoferrin-binding properties (Bitsaktsis *et al.*, 2012 ; Neeleman *et al.*, 1999; Ren *et al.*, 2003; Shaper *et al.*, 2004; Tu *et al.*, 1999). Moreover, it has been shown that pneumococcal surface protein A also plays a role in pneumococcal susceptibility. Underliyng mechanisms are actually not completely understood but depending on the type of PspA expressed by *S. pneumoniae*, strains are resistant or well-susceptible to some classes of antibiotics and not to other (Hotomi *et al.*, 2013). Based on this, PspA screening in patients, before administration of antimicrobials, would be an interesting way to predict treatment failure for some molecules and therefore improve the appropriateness of antibiotic choices to reduce further resistance.

Choline-binding proteins A (CbpA, also called SpsA or PspC) is known as an adhesin involved in pneumococcal attachment to eukaryotic membranes and abiotic surfaces, favoring cells infection and biofilm formation (Kadioglu *et al.*, 2008; Sanchez *et al.*, 2011b). The protein also possesses a protective role for bacteria by binding complement component C3 (Smith and Hostetter, 2000).

Neuraminidase A (Nan A) is a sialidase cleaving n-terminal osidic bond between sialic acid (also called n-acetylneuraminic) residues and other sugars, galactoses in most of cases. Sialic acids are present in human secretions such as saliva or milk but they are also present at the surface of viral, bacterial and eukaryotic cells (Trappetti *et al.*, 2009; Soong *et al.*, 2006; Lee *et al.*, 2007; Tram *et al.*, 1997). This cleavage allows the creation of "anchorage receptors" and makes these enzymes essential for pneumococcal airways colonization by allowing prokaryote-eucaryote bindings and for biofilm formation, by allowing attachment between bacteria (Parker *et al.*, 2009; Soong *et al.*, 2006). This enzyme also facilitates the pneumococcal colonization of the airways, such as in the nasopharynx, through mucus degradation (Mook-Kanamori *et al.*, 2011).

Figure 11



Sialic acid cleavage by the neuraminidase enzyme

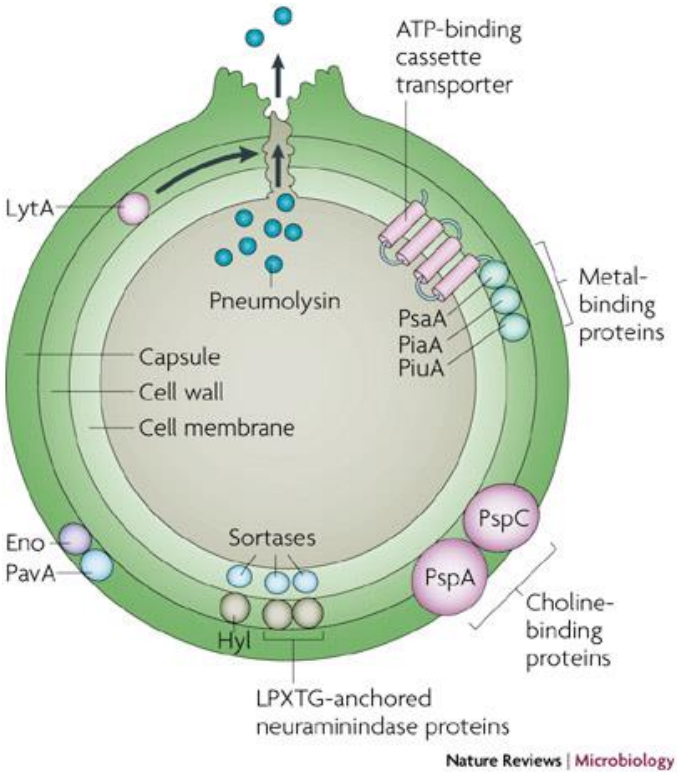
http://www.twiv.tv/virus-entry-into-cell

Membranous NADH oxidase (NOX) is an enzyme catalyzing the reduction of free molecular oxygen into water and therefore participating to bacterial well-balance by avoiding oxidative stress (Yu *et al.*, 2001). Moreover, it has be shown that this enzyme also have adhesive properties to nasopharynx and lung tissues, favoring the pneumococcal colonization process during infections (Muchnik *et al.*, 2013).

Autolysin A (LytA) is an amidase, cleaving n-acetylmuramoyl-l-alanine bonds of pneumocoocal peptidoglycans and therefore, playing several important roles in bacterial physiology (for e.g., in wall growth and turnover, cell separation in microorganisms and in the bacterial lysis process) and during infections by allowing the release of some virulence factors such as pneumolysin described here below (Jedrzejas, 2001; Kadioglu *et al.*, 2008; Orihuela *et al.*, 2004) and playing itself a role of virulence factor (Mellroth *et al.*, 2014). The enzyme is expressed by all pneumococcal strains and is therefore also used in *S. pneumoniae* diagnostic by making RT-PCR detection of lytA gene (Cvitkovic, V *et al.*, 2013).

Pneumolysin (Ply) is a 53kDa cholesterol-dependent cytolisin secreted by Gram positive bacteria, through the action of autolysin previously described. Their mode of action tells first binding to cholesterol of host cells membrane, followed by insertion into the target membrane to create pores (diameter : 260 Å) that leads to host cells dysfunctions and virulence (decrease of the ciliated bronchial cells beating, facilitating the spread of pneumococcal infections ; inhibition of the phagocyte respiratory burst ; induction of cytokine synthesis; CD4+ T cell activation and chemotaxis ; activation of the complement), causing finally lysis of the eukaryotic cell (Jedrzejas, 2001; Kadioglu *et al.*, 2004; Kadioglu *et al.*, 2008).

Figure 12



Pneumococcal virulence factors. the capsule; pneumococcal surface proteins A and C (PspA and PspC); the LPXTG-anchored neuraminidase proteins; hyaluronate lyase (Hyl); pneumococcal adhesion and virulence A (PavA); enolase (Eno); pneumolysin; autolysin A (LytA); metal-binding proteins pneumococcal surface antigen A (PsaA), pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA) (Kadioglu *et al.*, 2008).

2.3. Serotype

On the basis of their capsular polysaccharides, strains of *S. pneumoniae* can be classified into more than 90 serotypes (Weinberger *et al.*, 2010). Two nomenclatures (Danish and American) exist to define them. Actually, the Danish classification, distinguishing serotypes (for e.g. 6A and 6B) following structural and antigenic differences in polysaccharides is widely adopted, at the opposite of the American one, ranking serotypes by attributing them a number on the basis of their order of discovery (AlonsoDeVelasco *et al.*, 1995). Serotype is mainly determined with specific antibodies, using the Quellung reaction but molecular biology may also be used (Flamaing *et al.*, 2008; Moreno *et al.*, 2005).

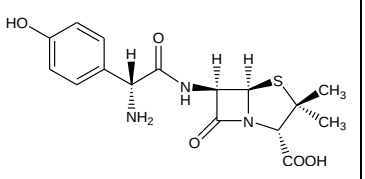
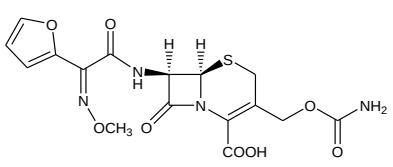
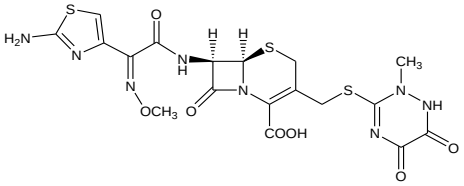
Some serotypes are reported as more invasive and causing acute infections such as pneumonia or sepsis (e.g. 1, 3, 5, 7F) when others are less invasive but more frequently found in chronic infections such as AECB or chronic otitis media (e.g. 3, 6A/B, 9N/V, 11A, 15A, 19F, 21, 23A, 33F, 35B) (Dagan *et al.*, 2013; Domenech *et al.*, 2012; Domenech *et al.*, 2013a). It seems that all pneumococcal serotypes do not have the same ability to produce biofilm. Serotypes 6B, 10A, 11A, 14, 15B and 33F are reported as high biofilm producers when serotypes/serogroups 1, 3, 4, 8, 12F, 38 are described as less productive.

2.4. *Streptococcus pneumoniae* antibiotic spectrum and resistance

Five antibiotic classes may be used to deal with infections caused by *S. pneumoniae*: β-lactams, macrolides, ketolides, fluoroquinolones and glycopeptides (Van Bambeke *et al.*, 2004). However, all of these are not actually recommended for clinical use or administrated to patients suffering from pneumococcal infections. This will be discussed here below within the description of each antibiotic class.

β-lactams consist of a broad class of antibiotics that includes penicillin and its derivates (for e.g. amoxicillin), cephalosporins (e.g. cefuroxime [second generation] and ceftriaxone [third generation]), carbapenems (e.g. meropenem, imipenem) and monobactams (e.g. aztreonam). All of these molecules are characterized by a β-lactam ring in their chemical structure. Figure 13 shows the three β-lactams used in this research work.

Figure 13

		
amoxicillin	**cefuroxime**	**ceftriaxone**

They all possess a bactericidal activity mediated by alterations in synthesis of the cell wall rich in peptidoglycans. In more detail, β-lactams are analogues of the D-Ala-D-Ala termination of the nascent peptidoglycan precursor, normally cleaved by transpeptidases called penicillin-binding proteins (PBPs) and performing the final transpeptidation step in the peptidoglycal synthesis. Through their structural similarity with PBPs substrate, these antibiotics are playing a role of suicide substrate disrupting wall synthesis by irreversible binding to the transpeptidases (Soares *et al.*, 2012).

On that basis, it is easy to understand that resistance to β-lactams occurs through alterations in pneumococcal penicillin-binding proteins, leading to a decreased antibiotic affinity for its target (Van Bambeke *et al.*, 2007). At the present time, the rate of pneumococcal resistance to β-lactams is between 6-10% in Belgium (Lismond *et al.*, 2012; Vanhoof *et al.*, 2010).

Concerning adverse-effects, I will only focus on those of penicillins and cephalosporines because these are the β-lactams subtypes that were used in my researches.

Penicillins, even at high dosage, are generally well tolerated. However, in some cases, their use can lead to allergies that may be serious and accompanied by mortality. This allergic process may tell 3 main steps: IgE-mediated early anaphylactic reaction (in the first minutes after intake and composed of rhinitis, erythema, bronchospasm, hypotension, choc), IgE-mediated delayed reaction (after a few hours) characterized by laryngeal oedema and finally, the late reaction with IgE and IgM-mediated rash, laryngeal and lip oedema and interstitial nephritis appearing within 3 days.

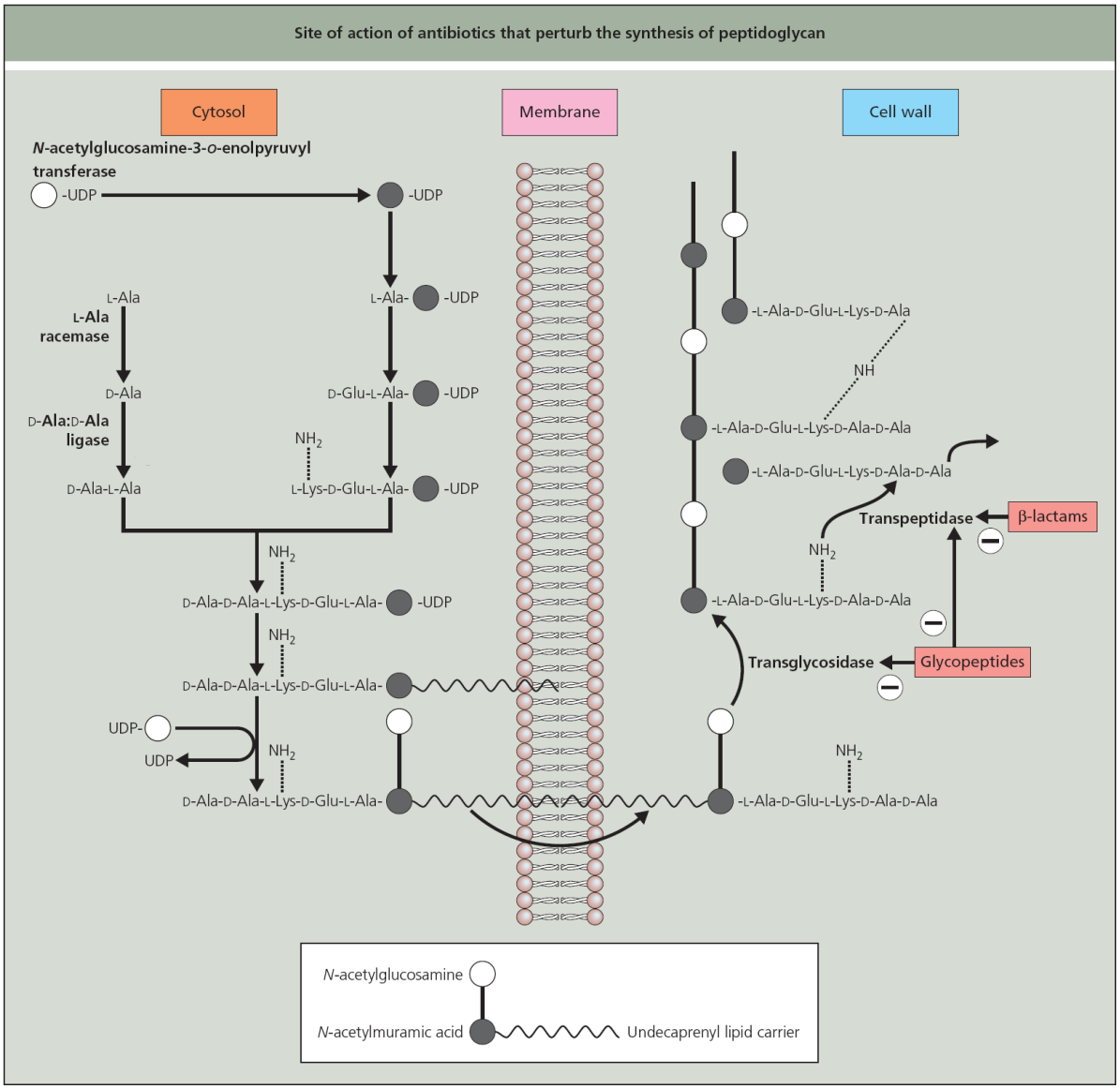
For cephalosporins, frequently administered by parenteral route, Ig3-meadiated allergic reactions are less frequent but there is a possible risk of thrombophlebitis at the injection site. Cefuroxime and ceftriaxone remain well safe but intake of other molecules such as cephaloridine or cefoperazone may respectively leads to renal toxicity and pseudomembranous colitis by *C. difficile* post-infection.

Glycopeptides are slowly bactericidal and, similar to β-lactams, act as inhibitors of the Gram positive wall synthesis. However, such illustrated in Figure 13, their mechanism of action is different. Indeed, through their aglycone fraction, these peptides are binding to the D-Ala-D-Ala termination of the peptidoglycan precursor and create a steric hindrance, blocking the access of the transpeptidase previously described to its target but also, the action of the transglycosidase responsible of the addition of new disaccharides-pentapeptide units to the growing polymer.

Glycopeptides (e.g. vancomycin) are not administered to patients suffering from pneumococcal infections, mainly because of their huge kidney- and oto-toxicity and the uncomfortable intravenous route of administration.

However, because of their fast rate of bacterial killing and their good intracellular activity already demonstrated *in vivo* for *S. pneumoniae* and in patients for other Gram positive species (e.g. *S. aureus* and Enterococci), they can become interesting candidates, after fluoroquinolones, for patients suffering from severe Gram positive infections (e.g. caused by multi-resistant strains) or being allergic to penicillins. This is especially true for lipoglycopeptides oritavancin and dalbavancin, actually in clinical development and presenting the best pharmacodynamic and pharmacokinetic properties in addition to lower incidence of adverse effects (Van Bambeke *et al.*, 2004).

Figure 14



Site of action of antibiotics that perturb the synthesis of peptidoglycan (Van Bambeke F, 2010)

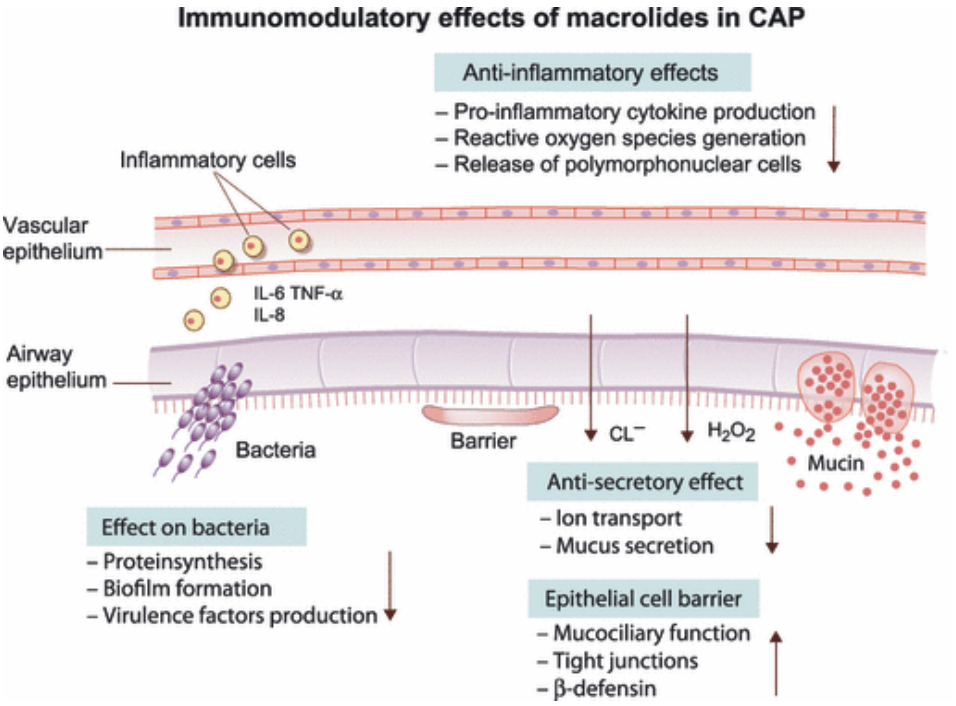
The peptidoglycan unit is formed in the cytosol of bacteria by binding to uridine diphosphate (UDP) *N* -acetylmuramic acid of a short peptide (the nature of which differs between bacteria). This precursor is then attached to a lipid carrier and added to *N* -acetylglucosamine before crossing the bacterial membrane. At the cell surface peptidoglycan units are reticulated by the action of transglycosylases (catalyzing the polymerization between sugars) and of transpeptidases (catalyzing the polymerization between peptidic chains). The antibiotics act as follows: fosfomycin is an analogue of phosphoenolpyruvate, the substrate of the *N* -acetylglucosamine-3- *O* -enolpyruvyl transferase synthesizing *N* -acetylmuramic acid from *N* -acetylglucosamine and phosphoenolpyruvate; cycloserine is an analogue of D -Ala and blocks the action of D -Ala racemase and D -Ala: D -Ala ligase; bacitracin inhibits the transmembrane transport of the precursor; vancomycin binds to D -Ala- D -Ala termini and thus inhibits the action of transglycosylases and transpeptidases; and β -lactams are analogs of D -Ala- D -Ala and suicide substrates for transpeptidases.

Macrolides are antibiotics characterized in their chemical structure by a lactone macrocyclic ring of 14 to 16 atoms to which one or more deoxy sugars, usually cladinose and desosamine may be attached (see figure 14 for structures of molecules used in this work). Their activity is mainly bacteriostatic and is mediated by the inhibition of bacterial protein synthesis after antibiotic binding to the ribosome, leading to blocking elongation of the nascent polypeptide by binding to the opening of the ribosomal exit tunnel (site P of the ribosome 50S subunit) causing a drop-off event leading to the accumulation of toxic peptidyl-transfer RNA (Soares *et al.*, 2012). Concerning adverse-effects, digestive intolerance is the most frequent side-effect reported for macrolides and is explained by the antibiotic binding to gut smooth muscles motilin receptors. Rarely, cholestatic hepatitis is observed.

Macrolide resistance is very frequent (30%, Vanhoof *et al.*, 2010) in Belgium and worldwide and is mediated, in *S. pneumoniae*, by three main mechanisms: (i) firstly, ribosome methylation by pneumococcal methylases coded by genes *ermB* (majority) and *ermA* (minority) leading to steric hindrance inhibiting macrolide binding (Roberts *et al.*, 1999). This mechanism confers cross-resistance to macrolides, lincosamides (e.g. clindamycin) and streptogramine B (MLS_B resistance phenotype) (Rosato *et al.*, 1999). The phenotypic expression of this resistance may consist of two types: constitutive, with resistance to 14-, 15- and 16-atoms macrolides and lesser susceptibility to ketolides (described here below), or inducible, with only resistance to 14-atoms molecules but full-susceptibility to 16-atoms macrolides and ketolides (Zhanel *et al.*, 2001); (ii) secondly, resistance to 14- and 15-atoms macrolides mediated by efflux. This resistance mechanism will be discussed further in more detail; thirdly, ribosome mutations leading to a decreased affinity of the antibiotic for its target.

In addition to their antibacterial activity, macrolides also have immunomodulatory properties observed in patients. Among them, we can mention the inhibition of the synthesis and/or secretion of pro-inflammatory cytokines, probably mediated by an effect of these drugs on the activation of transcription factors (Altenburg *et al.*, 2011; Giamarellos-Bourboulis, 2008). Moreover, it seems that macrolides promote alveolar macrophages differentiation and phagocytosis of apoptotic cells and therefore, avoid secondary inflammation linked to cell necrosis and release of virulent factors. Macrolides are also described as acting on neutrophiles, for example, by decreasing their chemotactic response to chemokines (Giamarellos-Bourboulis, 2008). The other inflammatory properties of macrolides on epithelia described in the review published by Meijvis and colleagues in 2012 are illustrated here below in Figure 15.

Figure 15



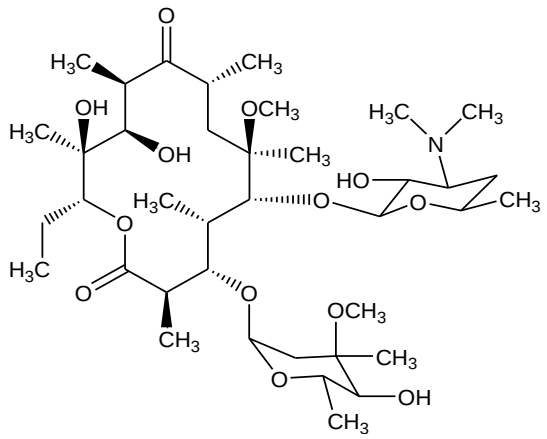
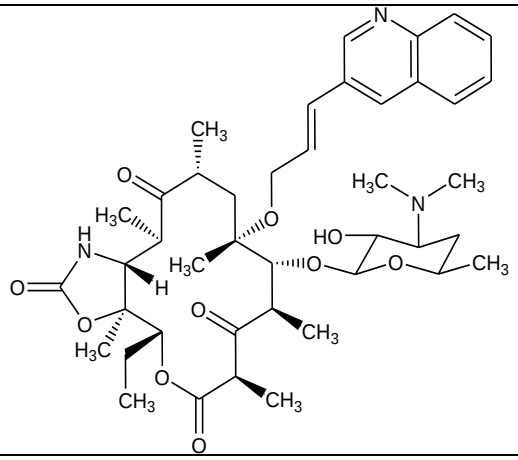
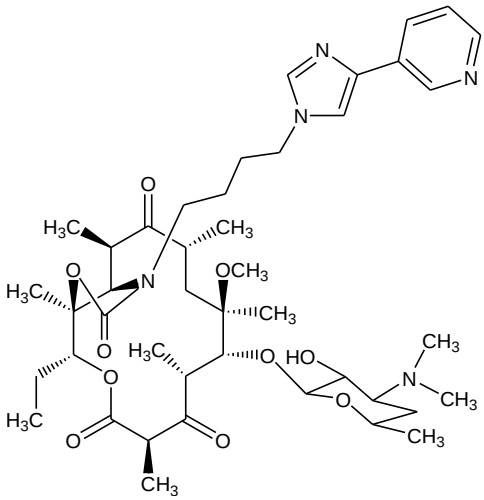
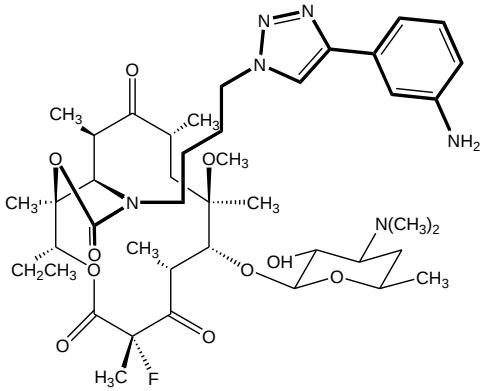
Schematic representation of the different macrolides immunomodulatory effects observed in a context of community-acquired pneumonia (CAP). (Meijvis *et al.*, 2012)

Ketolides are macrolide-derived antibiotics also working through the targeting of bacterial ribosome but with also a bactericidal activity. However, in their structure, (i) the macrolide's cladinose is substituted by a keto group leading to no induction of ribosome methylation and higher stability at acidic pH, (ii) a carbamate group is attached to the macrocyclic ring, increasing the activity and (iii) a lateral chain confers a two-site binding (at domains II, in addition to binding to domain V), a binding to methylated ribosomes, no recognition by efflux pumps and a better pharmacokinetic profile (Van Bambeke *et al.*, 2008). Actually, telithromycin is the only molecule available on the market worldwide. This molecule possesses the advantage of a very high susceptibility (0-2% in Belgium, Lismond *et al.*, 2012; Vanhoof *et al.*, 2010) and is rarely toxic but its adverse effects are very severe when they occur: hepatic and ocular toxicity, unconsciousness and *Myasthenia gravis* worsening. For this reason, it is almost never administered.

Figure 16 here below shows the chemical structures of the two macrolides (clarithromycin and azithromycin) and two ketolides (telithromycin and solithromycin) used in this work. Solithromycin is a new promising ketolide, currently in phase 3 of clinical development for community-acquired pneumonia (see http://www.clinicaltrials.gov- study NCT01756339) and presenting less toxicity than

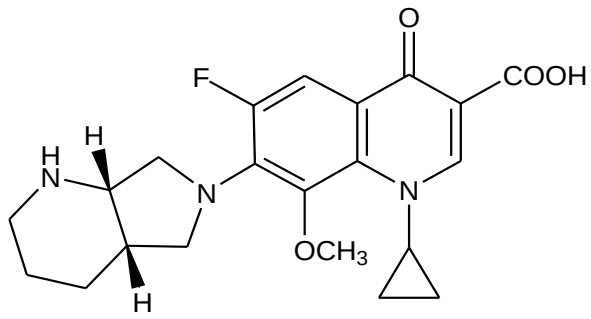
telitromycin, probably because of the lack, in its structure, of the pyridine-imidazole group responsible of *Myasthenia gravis* worsening through interaction with nicotinic acetylcholine receptors (Bertrand *et al.*, 2010).

Figure 16

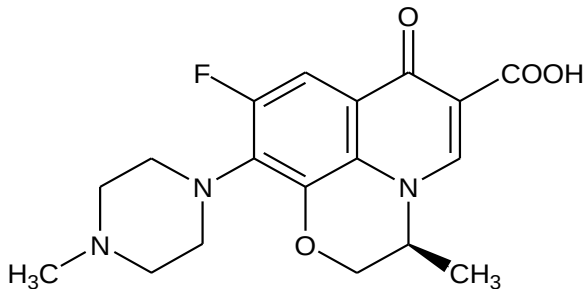
	
clarithromycin	**azithromycin**
	
telithromycin	**solithromycin**

Fluoroquinolones are synthetic bactericidal antibiotics binding to pneumococcal topoisomerase IV with thousand times more affinity than to eukaryotic enzymes and, therefore, inhibiting DNA unwinding during its replication. To do this, the antimicrobial molecule inserts itself between DNA strands and interferes with the DNA-topoisomerase IV complex through its lateral substituents and keto groups (see chemical structures of fluoroquinolones used in this work in Figure 17). Resistance to this antibiotic class is mediated by mutation in the target enzyme or overexpression of efflux pumps but remains still limited (Avrain *et al.*, 2007; Vanhoof *et al.*, 2010). For those reasons, ciprofloxacin and norfloxacin are no longer used in clinical practice. At the opposite, moxifloxacin, and sometimes levofloxacin, are used to treat adults patients but only in alternative to β-lactams because their adverse effects are frequent and severe (digestive and hepatic disorders; photo-, nephro-, chondro- and nervous toxicity).

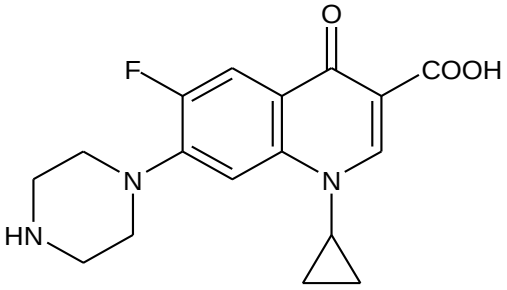
Figure 17



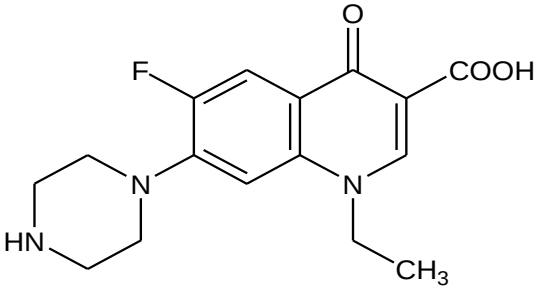
moxifloxacin



levofloxacin



ciprofloxacin



norfloxacin

Other antibiotic classes such as oxazolidinones (e.g. linezolid) are active against *S. pneumoniae* but almost never administered to patients because of their very important toxicity. For that reason, they are not described here in more detail.

2.5. Pharmacologic management of pneumococcal exacerbations of chronic bronchitis

The viral pathogens associated with AECB are influenza, parainfluenza, rhinovirus, coronavirus, adenovirus and respiratory syncytial virus. Concerning bacterial causes, such as previously mentioned, *Streptococcus pneumoniae* is one of the most prevalent bacterial causes of AECB in patients suffering from mild, intermediate and severe COPD (Eller, 1998; Albertson and Chan, 2009).

Concerning the therapeutic management of AECB, amoxicillin is the first choice of antibiotic treatment recommended both by the Belgian guidelines and in the literature for the management of bacterial infections occurring in COPD patients with no allergy to penicillins and if the incidence of AECB is inferior to 3 per year (Belgian Antibiotic Policy Coordination Committee (BAPCOC), 2012; Van Bambeke *et al.*, 2007). For patients developing more frequently exacerbations, recommendations are in favor of alternations between amoxicillin and moxifloxacin. These choices are taking the antibiotic safety profile and the resistance incidence into account (Belgian Antibiotic Policy Coordination Committee (BAPCOC), 2012 ; Van Bambeke *et al.*, 2007). If only bactericidal activity and quality of life are considered, moxifloxacin seems to give slightly higher results than amoxicillin (Miravitlles, 2007; Miravitlles *et al.*, 2010).

Additionally, prophylactic influenza and pneumococcal vaccinations are currently recommended for all persons suffering from COPD (Mirsaeidi *et al.*, 2014; Varkey *et al.*, 2009). However, the real benefits of these vaccines and their impact on the development of infections remain controversial and vary depending on the vaccine administered.

Concerning the influenza vaccination, observational studies including a large number of individuals (> 100 000) have shown that the influenza vaccine reduces by more than 50% the rate of hospitalization and by 70% the risk of mortality in elderly patients presenting chronic lung disease (Poole *et al.*, 2006; Nichol *et al.*, 1999). Influenza vaccination has been demonstrated not only as effective at reducing acute exacerbations of COPD, but is also a highly cost-effective intervention (Varkey *et al.*, 2009).

Concerning clinical studies conducted on the utility of pneumococcal vaccination in COPD patients, nonrandomized observational studies demonstrate strong evidence that pneumococcal vaccine protects healthy persons against invasive pneumococcal disease such as bacteremia. Observational data also provide evidence that pneumococcal vaccine confers protection in the elderly and persons with COPD (Varkey *et al.*, 2009).

Randomized controlled trials, however, have consistently failed to demonstrate a statistically significant benefit (in terms of rates of hospitalization, emergency department visits, pneumonia, mortality, and length of hospital stay) of pneumococcal vaccination in patients with COPD (Varkey *et al.*, 2009). For instance, the large single-blinded randomized controlled trial published by Alfageme

and colleagues in 2006 has shown that the administration of the 23-valent pneumococcal capsular polysaccharide vaccine (PPV-23) in COPD patients failed to demonstrate a difference in time to first pneumonia and mortality rate (Alfageme *et al.*, 2006). However, it seems that the level of protection afforded by the 23-valent pneumococcal vaccine in depending on the type of infection considered. As an example, the American study published by Musher and colleagues in 2006 showed that, despite the fact that rates of prophylactic PPV-23 vaccination were higher (60% *vs.* 39%) in patients suffering from non-bacteremic pneumococcal infections (e.g. AECB) than in those presenting bacteremic pneumonia or any invasive pneumococcal infection, no or only moderate protection against pneumococcal AECB episodes was provided by vaccination (Musher *et al.*, 2006). Some explanation for the above observations can be the incomplete coverage of all pneumococcal serotypes, providing no protection against uncovered serotypes with, perhaps also, a more substantial involvement of some pneumococcal serotypes in some infections types than others.

We have also to notice that vaccination rates mentioned in this last study for COPD patients strongly differ from Belgian recent data. Indeed, it seems that only a small proportion, approximately 5%, of Belgian patients suffering from chronic bronchitis have been vaccinated (Verhaegen *et al.*, 2014). This may suggest that the current medical management and follow-up of COPD patients is not optimal in our country. It also highlights the importance and the need of local medical care and of being under the care of a general practitioner.

2.6. Pneumococcal efflux pumps

Efflux pumps are ubiquitous transmembrane protein transporters present in prokaryotic and eukaryotic cells. They assure physiological and protective roles by making extrusion of poorly diffusible molecules or toxic endogenous compounds produced by cellular metabolism (Van Bambeke *et al.*, 2003) but antibiotic efflux also occurs for some molecules.

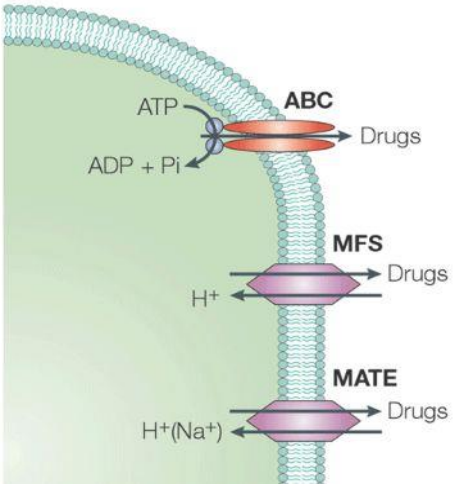
Such as illustrated in Figure 18 here below, three types of antibiotic efflux pumps have been described in the literature for *S. pneumoniae*:

- Major Facilitator Superfamily pumps (MFS) represented by MefE and PmrA and working using energy of the proton motive force (Wierzbowski *et al.*, 2005).

- ATP Binding Cassette (ABC) pumps represented by the heterodimer PatA/PatB and using the energy of adenine triphosphate (ATP) hydrolysis (Boncoeur *et al.*, 2012).

- Na^+/drug antiporters called Multidrug And Toxic coumpounds Extrusion (MATE) pumps (Hashimoto *et al.*, 2013).

Figure 18



Different types of efflux pumps actually described for *S. pneumoniae* (Krulwich *et al.*, 2005)

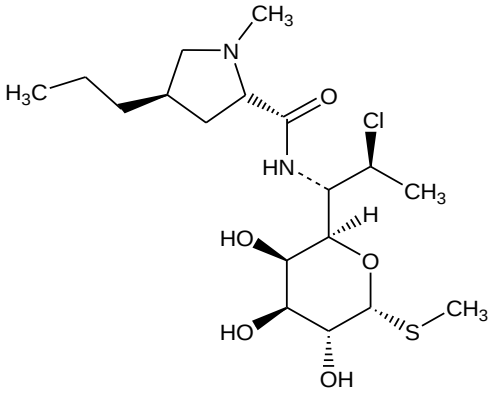
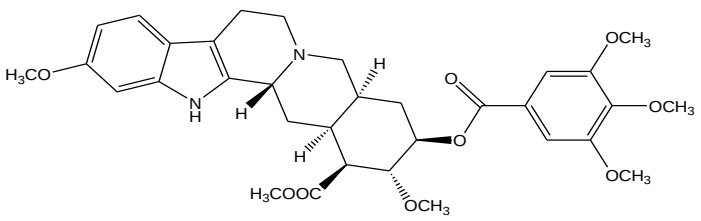
Efflux pump MefE extrudes 14- (e.g. erythromycin, clarithromycin) and 15-atoms (e.g. azithromycin) macrolides (Wierzbowski *et al.*, 2005). The other MFS protein, PmrA, is responsible for the efflux of norfloxacin, a fluoroquinolone (Gill *et al.*, 1999). Concerning the heterodimer PatA/PatB, it takes norfloxacin and ciprofloxacin in charge (Marrer *et al.*, 2006) and the MATE family DinF transport

system (SP1939) is described as probably causing efflux of 3 fluoroquinolones, levofloxacin, ciprofloxacin and moxifloxacin because mutants which do not express it show an increased susceptibility to these molecules (Tocci *et al.*, 2013).

Because these pumps decrease cytoplasm antibiotic concentration, their overexpression leads to low-level bacterial resistance (Marrer *et al.*, 2006; Avrain *et al.*, 2007; Garvey and Piddock, 2008), but bacteria in which efflux proteins are overexpressed may survive to antibiotic treatments and therefore develop and select mutations in genes coding for antibiotic targets, leading to high-level resistance (Sun *et al.*, 2014). In the same way, efflux overexpression may be a resistance mechanism developed in addition to others, such as a single mutation in antibiotic target. This mutation will probably have only minor impact on the strain resistance profile but, if the intracellular antibiotic concentration is decreased through efflux, the MICs measured may however be higher than the clinical breakpoint (Van Bambeke *et al.*, 2003).

The presence of phenotypic resistance mediated by efflux is determined in two different ways, depending on the antibiotic class investigated. For macrolides, it's determined by comparing the Minimal inhibitory concentrations (MICs) of strains resistant following EUCAST breakpoints with the MIC measured for clindamycin, a lincosamide for which cross-resistance is observed with macrolides but that is not substrate of efflux pumps (Lismond *et al.*, 2012). For fluoroquinolones, phenotypic efflux is highlighted by making MIC determination in absence versus presence of reserpine, an non-antibiotic efflux pump inhibitor (Lismond *et al.*, 2012; Wierzbowski *et al.*, 2005). The overexpression of genes coding for efflux pumps taking macrolides and fluoroquinolones in charge is assessed by RT-PCR.

Figure 19

	
clindamycin	**reserpine**

An important characteristic of efflux pumps is there inducible character by subinhibitory concentrations of their own antibiotic substrates or other compounds. This was demonstrated in *in vitro* cultures of *S. pneumoniae* with the induction of operon *mef(E)-mel* by erythromycin. This is related to specific interactions of the macrolide C-5 saccharide with the ribosome that alleviate transcriptional attenuation of *mef(E)-mel* (Chancey *et al.*, 2011). Similar results on *S. pneumoniae* have been obtained in our laboratory by Laetitia Avrain who demonstrated that subinhibitory concentrations of ciprofloxacin could induce the overexpression of genes *patA* and *patB* but not *pmrA* (Avrain *et al.*, 2007). These results were confirmed by another researcher, Farid ElGarch, who published results showing that the overexpression of genes *patA* and *patB* could be induced by five fluoroquinolones (norfloxacin, ciprofloxacin, levofloxacin, moxifloxacin and gemifloxacin) but that *pmrA* expression remains unaffected (El Garch *et al.*, 2010).

Other research groups have shown that non-antibiotic drugs are also able to modulate antibiotic efflux in different bacterial species. Thus, it has been demonstrated that the expression of efflux pumps could be induced by salicylates in *S. aureus* (Riordan *et al.*, 2007), *S. typhimurium* (Hartog *et al.*, 2010) and Enterococci (Giuliodori *et al.*, 2007) and by diazepam in this last bacterial species (Giuliodori *et al.*, 2007; Tavio *et al.*, 2004). At the opposite, L-thioridazine, an antipsychotic drug, inhibits fluoroquinolone and macrolide efflux in *S. pyogenes* and *S. aureus* (Kristiansen *et al.*, 2007), while omeprazole, a proton pump inhibitor, significantly improves cipro- and norfloxacin activities against Staphylococci by inhibiting their efflux (Aeschlimann *et al.*, 1999). Finally, fluoxetine, a selective serotonin reuptake inhibitor, has also been characterized as inhibiting *S. aureus* efflux proteins (Kaatz *et al.*, 2003).

2.7. Pneumococcal special life modes favoring antibiotic failure

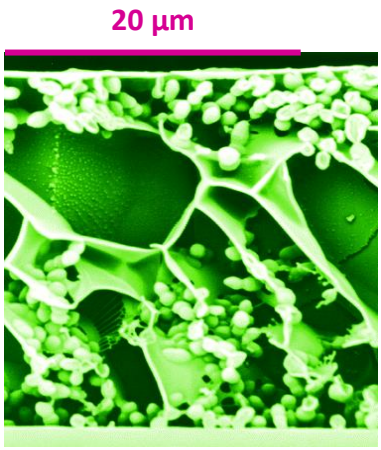
Pneumococcal intracellular persistence has already been described in biopsies of patients suffering from chronic otitis media (Coates *et al.*, 2008). This special life mode, such as growth within biofilms is known to favor antibiotic failure and persistence of infections (Moscoso *et al.*, 2009; Simoes, 2011; Van Bambeke *et al.*, 2006). Antibiotic activity on pneumococci growing within biofilms was the main point of interest in this work. For that reason, biofilms and especially those produced by *S. pneumoniae* are described below.

3. Pneumococcal biofilms

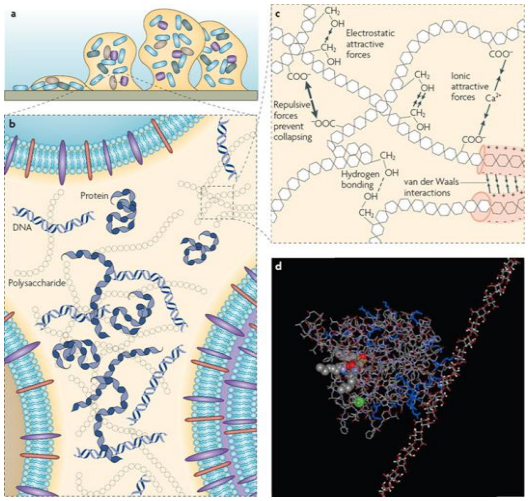
3.1. Definition and composition

Biofilms are defined as three-dimensional communities of sessile microorganisms adhering to a surface or interface and embedded in a structured matrix called extracellular polymeric substance (EPS), most often hydrated and containing polysaccharides, proteins, extracellular DNA and signaling molecules (Moscoso *et al.*, 2006; Moscoso *et al.*, 2009; Vlastarakos *et al.*, 2007). This can explain why negative charges are present within the matrix. They are responsible of chemical interactions between bacteria and matrix components and allow the biofilm thickness quantification through staining with cationic dyes such as crystal violet (Christensen *et al.*, 1982). Figure 20 shows the honeycomb structure of the matrix in which pneumococci are embedded (left) and the chemical forces existing within the biofilm matrix (right).

Figure 20



Low Temperature Scanning Electron Microscopy (LTSEM) of a *S. pneumoniae* R6 biofilm formed on a glass surface (Moscoso *et al.*, 2006).



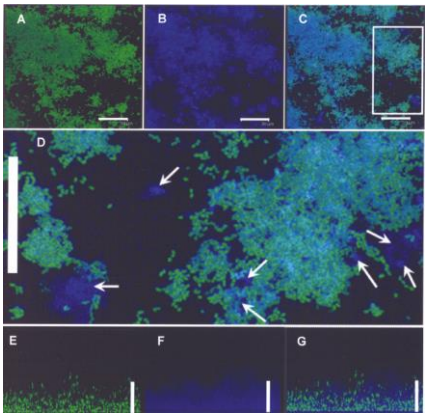
The extracellular polymeric substances matrix at different dimensions. **a** | A model of a bacterial biofilm attached to a solid surface. Biofilm formation starts with the attachment of a cell to a surface. A microcolony forms through division of the bacterium, and production of the biofilm matrix is initiated. Other bacteria can then be recruited as the biofilm expands owing to cell division and the further production of matrix components. **b** | The major matrix components — polysaccharides, proteins and DNA — are distributed between the cells in a non-homogeneous pattern, setting up differences between regions of the matrix. **c** | The classes of weak physicochemical interactions and the entanglement of biopolymers that dominate the stability of the EPS matrix[47]. **d** | A molecular modeling simulation of the interaction between the exopolysaccharide alginate (right) and the extracellular enzyme lipase (left) of *Pseudomonas aeruginosa* in aqueous solution. The starting structure for the simulation of the lipase protein was obtained from the Protein Data Bank. The coloured spheres represent 1,2-dioctylcarbamoyl-glycero-3-*O*-octylphosphonate in the lipase active site (which was present as part of the crystal structure), except for the green sphere, which represents a Ca^{2+} ion. The aggregate is stabilized by the interaction of the positively charged amino acids arginine and histidine (indicated in blue) with the polyanionic alginate. Water molecules are not shown. Image courtesy of H. Kuhn, CAM-D Technologies, Essen, Germany. (Flemming and Wingender, 2010).

Biofilm pneumococci have highly modified protein profiles versus their planktonic counterparts (Sanchez *et al.*, 2011a). Concerning biofilm, the chemistry of EPS differs between Gram negative and positive bacteria. For example, the Gram negative bacterial species *P. aeruginosa* produces alginate (Wiens *et al.*, 2014) when our Gram positive of interest, *S. pneumoniae*, does not. The composition of the pneumococcal extracellular matrix has already been widely investigated, mainly through microscopic visualization of specific compounds after fluorescent labelling, GC-MS polysaccharides identification and analyze of proteins-DNA complexes by agarose gel electrophoresis (Domenech *et al.*, 2013b).

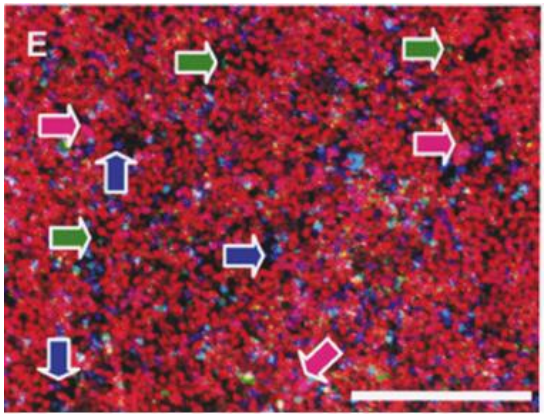
It could so be established that pneumococcal choline-binding proteins, namely LytA *N*-acetylmuramoyl-L-alanine amidase, LytB glucosaminidase, lytC lysozyme, CbpA, PcpA putative adhesine and PspA are present within the matrix, involved in biofilm formation and that strong binding exist between DNA and LytC. The formation of that complex requires Mg^{2+}, showing that ions are also present in the matrix and play an important role (Trappetti *et al.*, 2011b). Matrix polysaccharides are also different to the capsular polysaccharides (e.g. β-1, 3 and β-1, 4 polysaccharides such as chitin and cellulose and specifically labeled by Calcofluor in EPS) and their distribution through the matrix is irregular.

Fluorescent labeling allows for microscopic colocalization of matrix components using different probes. Living and dead bacteria are usually visualized using the LIVE/DEAD® Bacterial Viability Kit (*Bac*Light™) composed of two nucleic acid-binding stains: SYTO 9 and propidium iodide. SYTO 9 penetrates all bacterial membranes and stains the cells in green, while propidium iodide only penetrates cells with damaged membranes, and the combination of the two stains produces red fluorescing cells (Boulos *et al.*, 1999). Extracellular DNA can also be marked with these probes and by other markers including ethidium bromide, DAPI (4', 6-diaminido-2-phenylindole), and DDAO [7-hydroxy-9H-(1, 3-dichloro-9, 9-dimethylacridin-2-one)]. Matrix polysaccharides such as chitin can be specifically labeled using Calcofluor white M2R. The use of other specific markers such as Alexa-conjugate lectins may also be used to obtain accurate identification of some sugars of interest (Domenech *et al.*, 2013b). Indeed, matrix labeling with WGA, specific for GlcNAc and Neu5Ac and with soybean agglutinin, specific for Gal and GalNAc enabled researchers to detect these sugars in EPS. Similarly, the use of other lectins (e.g.peanut agglutinin specific of terminal residues of β-Gal, such as Galβ-1, 3-GalNAc; concanavalin A, specific for Man and Glc and dispersin B, hydrolysing the polysaccharide intercellular adhesin [PIA] and its derivates present in staphylococcal and enterococcal biofilms) gave negative results indicating that these polysaccharides are not present in the matrix of pneumococcal biofilms.

Figure 21



(a) Bacterial cells (strain P040, an R6 derivative expressing the green fluorescent protein); (b) The same biofilm stained with Calcofluor white M2R labeling polysaccharides; (c) A merger of the above two channels; (d) An enlargement, of the area marked with a rectangle in c, in which the arrows indicate Calcofluor-labelled regions that lack bacteria; (e-g) Side views of (a), (b) and (c) respectively. Scale bars = 25 µm. (Domenech *et al.*, 2013b).

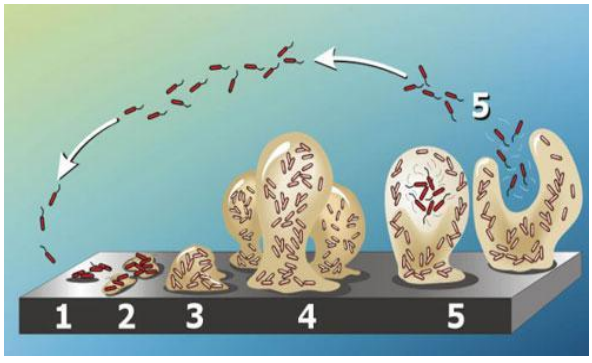


SYTO 60 (red) indicates R6 cells marked through their DNA and carbohydrates are labeled with or to carbohydrate SBA (green) and WGA (blue). Pink arrows indicate colocalization of the three fluorophores. Images are maximum projections of a series of *x–y* sections. Scale bars = 30 µm. (Domenech *et al.*, 2013b).

3.2. Biofilm development

Biofilm formation, illustrated here below is a 5-step dynamic process regulated by the Quorum sensing and composed of primary and reversible attachment of bacteria to a surface mediated by electrostatic attraction, followed by irreversible adhesion allowed by the action of adhesins. When bacteria are sticked on their support, they are intensively producing a matrix called extracellular polymeric substance (EPS) (Gilbert *et al.*, 2002). The fourth step is the maturation of the biofilm structure characterized by cell intensive division, formation of water channels, increase of the matrix amount, and association of secondary colonizers joining the structure. Nutrients, anoxic and acidic gradients exists though the matrix dept (Hall-Stoodley and Stoodley, 2009). Finally, the last step consists in the release of bacteria from mature biofilm, which recover a plancktonic state and participate to the colonization process (Vlastarakos *et al.*, 2007).

Figure 22



Biofilm development main steps

http://www.genomenewsnetwork.org/articles/06_02/biofilms.shtml

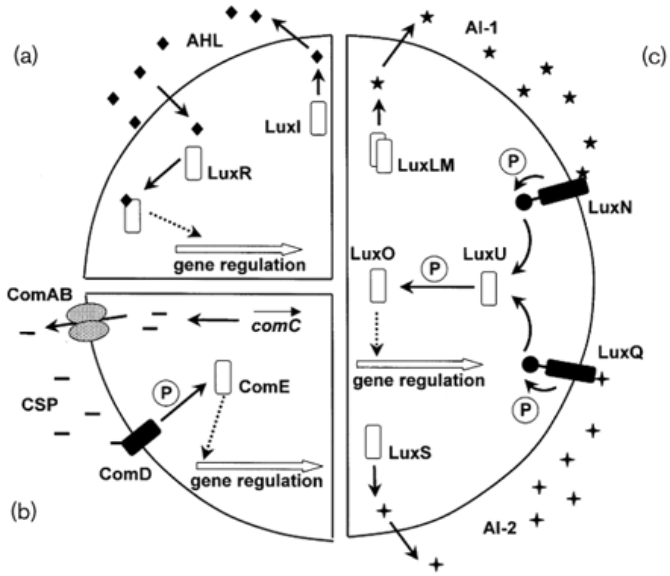
Quorum Sensing in pneumococcal biofilms

Quorum sensing (QS) is a physiological process developed by bacteria to communicate with each other and regulate their cooperative activities (e.g. biofilm production), in which bacterial cells produce, detect and respond to signal molecules. It's well known that the colonization process occurring during bacterial infections required a critical cell density. Since Quorum Sensing leads to cell-cell communication, its implication in infections is not surprising (Li and Tian, 2012).

Bacteria using Quorum sensing communication are producing signal molecules called auto-inducers or pheromones. The presence of auto-inducers is detected by intramembrane and intracellular receptors that bind them. Activated receptors recognize some DNA sequences of genes regulated by Quorum sensing and activate or down-regulate their expression. Among auto-inducers, we can mention N-acetyl homoserine lactones (AHL) in *P. aeruginosa* biofilms, and for *S. pneumoniae*, cyclic peptides (e.g. the autoinducer 2 [AI-2], synthetized by the enzyme Lux S from S-adénosine homocystéine), and the competence stimulating peptide [CSP]). Quorum sensing pathways differ between bacterial species and are divided into 3 classes, as partly illustrated in a simplified fashion here below in Figure 23 (McNab and Lamont, 2003; Li and Tian, 2012; Cook and Federle, 2014) :

(1) LuxI/LuxR–type quorum sensing in Gram-negative bacteria, which use AHL as signal molecules.
(2) Pheromones systems present in Gram-positive bacteria and including (a) the RNPP family of regulators, (b) Agr-type cyclical pheromones, (c) peptides with double glycine (Gly-Gly) processing motifs to which the oligopeptide-two-component-type competence quorum sensing system, acting through CSP binding to receptors ComD, belongs and, (d) regulators of the Rgg family.
(3) *luxS*-encoded autoinducers (e.g. AI-2) in both Gram-negative and Gram-positive bacteria.

Figure 23



Schematic diagrams of representative quorum-sensing systems (McNab and Lamont, 2003)

(a) LuxI/R quorum sensing. LuxI autoinducer synthase produces AHL molecules, which freely diffuse across the cytoplasmic membrane. At a critical concentration, AHL binds LuxR, generating an active response regulator that activates transcription of target genes. (b) Peptide-mediated quorum sensing in development of competence. The *comC* locus encodes a peptide pheromone precursor that is secreted as a mature signaling peptide (CSP) by a dedicated ABC transporter (ComAB). At a critical concentration, CSP binds to, and induces autophosphorylation of, the cognate histidine kinase (ComD) at the cytoplasmic membrane. The kinase subsequently phosphorylates and activates the response regulator (ComE). (c) Quorum sensing in *V. harveyi*. Two quorum-sensing circuits integrate cell-density information at the shared LuxU phosphorelay component. AI-1 and AI-2 are detected at the cell surface by LuxN and LuxQ, respectively (the periplasmic LuxP protein that is involved in recognition of AI-2 is not shown for simplicity).

Streptococcus pneumoniae biofilm formation is regulated by Quorum sensing systems ComC and LuxS-AI-2 (Vidal *et al.*, 2013). *In vitro* models have already shown that LuxS-AI-2 is mainly involved in early biofilm attachment (first step of formation), when the implication of system ComC has a major function in stabilizing older structures Vidal *et al.*, 2013; Trappetti *et al.*, 2011b).

One of their main functions is to regulate biofilm matrix production (Gilbert *et al.*, 2002) and autolysis. This process consists in noncompetent cells lysis (also called fratricide), release of eDNA, essential for biofilm formation and bacterial competence in supernatant (Vidal *et al.*, 2013; Trappetti *et al.*, 2011b).

Quorum sensing in *S. pneumoniae* may also be regulated itself. For example, Fe (III) upregulates LuxS expression (Trappetti *et al.*, 2011b) and antibodies produced in reaction to pneumococcal capsular polysaccharide-based vaccines enhance competence-induced fatricide (Yano *et al.*, 2011).

Other modulators and regulators of the biofilm formation

Among regulators of *S. pneumoniae* tissue colonization through biofilm formation, bacterial contact with epithelial cells takes, of course, a predominant place. It has been shown that abiotic surfaces provide less early biofilm formation for some pneumococcal strains (e.g. with already decreased PspA expression) (Vidal *et al.*, 2013; Marks *et al.*, 2012).

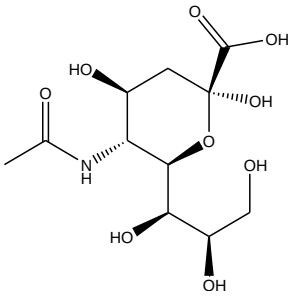
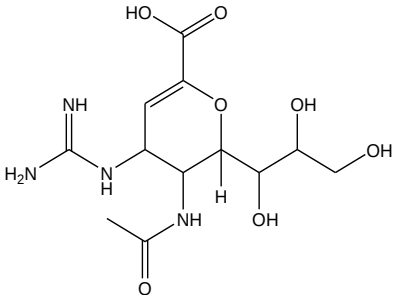
Attachment of *S. pneumoniae* to eukaryotic cells is mediated by adhesive and non-adhesive proteins activity. As an example, the Pneumococcal serine-rich repeat Protein (PsrP) mediate bacterial binding to Keratine10 of the lung cell surface and intrabacterial interactions through amino acids 273-341 located in its Basic Region Domain (Sanchez *et al.*, 2010; Moscoso *et al.*, 2006).

Another pneumococcal protein, the Neuraminidase A (NanA), is involved in initial colonization of the airways by participating to bacterial binding to epithelial surfaces and biofilm production through the cleavage of a sugar called sialic acid or n-acetylneuraminic acid, illustrated in Figure 24 (Soong *et al.*, 2006). Procaryotic and eukaryotic cells are highly sialylated (Lewis *et al.*, 2004). By cleaving sialic acid residues, NanA induces pneumococcal adherence between bacteria and to epithelia. This occurs through the modification of bacterial or host glycoconjugates and the exposition of binding receptors for adhesion (Brittan *et al.*, 2012; Parker *et al.*, 2009). Sialic acid at concentrations similar to those found in human saliva is enhancing *S.pneumoniae* biofilm formation through inducing the expression of enzyme NanA and mutations in gene *nanA* are accompanied by less biofilm formation observed *in vitro* (Trappetti *et al.*, 2009 ; Parker *et al.*, 2009). Similarly, neuraminidase inhibitors, used to target the viral Influenza neuraminidase, are also active on the pneumococcal NanA and modulate the

biofilm formation (Trappetti *et al.*, 2009). The structure of zanamivir, the neuraminidase inhibitor used in this work, is illustrated in Figure 24.

Finally, a virulent factor, Pneumolysin, seems to modulate very early attachment and biofilm formation by regulating Quorum sensing luxS/AI-2 system (Shak *et al.*, 2013).

Figure 24

	
sialic acid	**zanamivir**

Considering now modulations of the biofilm formation by toxic compounds, long (16h) exposure of *S.pneumoniae* to cigarette smoke and nicotine, at concentrations relevant of those found in smokers airways, leads to an increase of the bacterial adhesion and biofilm matrix production. It seems that this occurs through the induction of quorum sensing pathways and the inactivation, probably mediated by smoke-related oxidative stress, of the pneumolysin, a major toxin involved in bacterial pore formation. This may also have some implication on the host immune response if we take into account that pores formed by this enzyme stimulate the production of IL-8 and early influx of neutrophils that contribute to early control of colonization (Mutepe *et al.*, 2013). Increase in biofilm formation and adhesion have been confirmed for biofilms made by other bacterial species such as *P. aeruginosa* (Antunes *et al.*, 2012, *S. mutans* (Huang *et al.*, 2012), *S. aureus* (Kulkarni *et al.*, 2012). However, it seems that this increase of biomass production, mediated by bacterial exposure to tobacco, may be reversed overtime when prokaryotic cells are cultivated during a sufficient period in the absence of exposure to tobacco smoke (Goldstein-Daruech *et al.*, 2011).

3.3. Biofilm implication in the infection process

It is actually well recognized that 60% of bacterial infections and up to 80% of chronic infections involve bacterial growth within biofilms (Moscoso *et al.*, 2009). Their presence has already been demonstrated *in vivo* (Chaney *et al.*, 2011; Post, 2001) in sputum, bronchoalveolar lavage (BAL) and lung biopsies of patients suffering from cystic fibrosis or chronic otitis media (Hall-Stoodley and Stoodley, 2009), such as on tympanostomy tubes (Post, 2001), cochlear implants, and sinus stents or tissue samples (Vlastarakos *et al.*, 2007; Zernotti *et al.*, 2010; Post, 2001).

This special life mode is characterized by increased resistance to drastic living conditions (alterations in nutrients, high cell density, temperature, pH and osmotic stresses), host defense cells and to antimicrobials (Lopez *et al.*, 2010; Fux *et al.*, 2005; Hall-Stoodley and Stoodley, 2009; Roveta *et al.*, 2007). Concerning its impact on antibiotic resistance, different interesting mechanisms are proposed:

I) Direct interactions between the components of the extracellular polymeric substance (EPS) and antibiotics affecting their availability through (i) limited diffusion (matrix plays a barrier protective role) mediated by biofilm thickness, (ii) chemical interactions with the EPS molecules. Indeed, polymers of the extracellular matrix may act as an ion exchange resin and actively filter out strongly charged molecules. Poor penetration through anionic matrices might, therefore, be a phenomenon restricting antibiotic diffusion and activity. Finally, (iii) matrix-retained bacterial enzymes may also be responsible of antibiotic inactivation in biofilms made by some bacterial species. A good example of that are beta-lactamases produced by Gram negative bacteria. These proteins, which may be secreted in the extracellular compartment and sequestered in the matrix, can lead to the inactivation of β-lactams used to eradicate biofilms (Gilbert *et al.*, 2002; Ciofu, 2003; Simoes, 2011; Lopez *et al.*, 2010).

II) A cellular dormant state within biofilm depth mediated by an altered (poorer) microenvironment and leading to reduced cell growth and division. Because several antibiotics are targeting DNA replication, protein synthesis or interfere with cell proliferation through altering the production of essential bacterial components (*e.g.* β-lactams inhibit cell wall synthesis), the metabolic transition to a "no growth state" characterized by no more proliferation and therefore, a down regulation of all processes mentioned here above, will be accompanied by an increased resistance to antibiotics.

III) Adoption of "stress resistant phenotypes" induced by environmental stress or high cell density. They can be of a different nature. Firstly, the matrix barrier role, limiting antibiotic diffusion, decreases antibiotics amounts in deep biofilms layers until subinhibitory concentrations which may induce the emergence of antibiotic efflux phenotypes (Maira-Litran *et al.*, 2000). Secondly, bacteria may adopt some attachment-specific phenotypes characterized by a faster adhesion to supports,

induced by the antibiotic pressure (Gilbert *et al.*, 2002). Finally, in some conditions, damaged bacterial cells are able to undergo apoptosis. This is the definition of "persister cells". After the removal of the stress condition, these persistors will grow rapidly in the presence of nutrients released from their lysed community partners and so restore the bacterial community (Simoes, 2011 ; Gilbert *et al.*, 2002; Lewis, 2007; Lewis, 2000).

IV) Increased frequency in mutations, favored by the higher density of bacterial cells within biofilms compared to planktonic cultures, and leading to faster acquisition of resistance to antibiotics (Blazquez, 2003 ; Conibear *et al.*, 2009).

V) More recently, authors have demonstrated the existence of biofilm-specific antimicrobial resistance genes. It seems that their expression is not essential for biofilm formation but decreases bacterial susceptibility to antibiotics by encoding for efflux pumps only expressed in sessile cells. Currently, *Pseudomonas aeruginosa* is the bacterial species for which the implication of these genes is the best characterized (Zhang *et al.*, 2013).

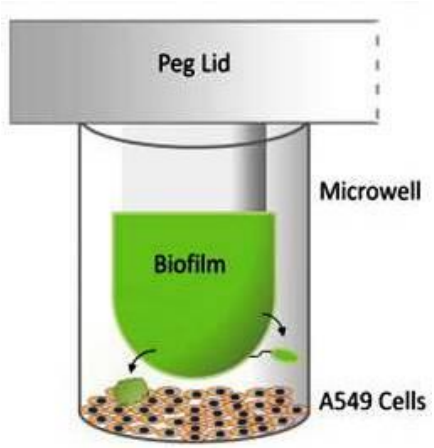
In addition to this, biofilms are involved in persistence of infections and tissue colonization through their role of bacterial reservoir. To address this problem, *in vitro* and *in vivo* models of bacterial and fungal biofilms have been developed over the last twenty years. They are currently used to investigate the therapeutic or prophylactic activities of antibiotics and new anti-biofilm agents towards cells living within the matrix or towards EPS itself through pharmacodynamic studies. The different types of existing models are described in the following paragraph.

3.4. *In vitro* biofilm static models

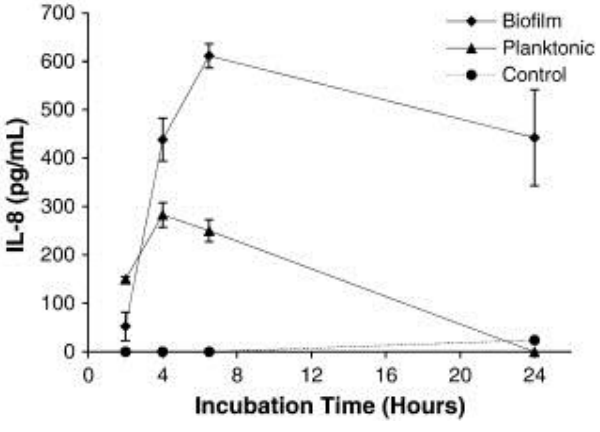
The microtiter plate model is actually the most-frequently used support for static growth of *in vitro* biofilms. We even used it for our experiments. The main advantages of this model are its cheapness, the opportunity to test a large number of different culture conditions at the same time and to use different types of plates coating to examine their influence on biofilm development overtime (Coenye and Nelis, 2010).

Among the different variations of microtiter plate models, the Calgary Biofilm Model developed by Ceri and colleagues (Ceri *et al.*, 1999) can be mentioned. In this system, biofilm develops on pegs attached to the lid of 96-well plates and immersed in the growth medium. Using this technique, biofilms can therefore been transferred to new microplates overtime. This model is mainly used to investigate the impact of successive treatments with different antimicrobial agents on biofilm eradication (Coenye and Nelis, 2010). To investigate the interactions existing between the innate immune system responses elicited by host epithelial cells and bacteria, Bowler and colleagues recently developed a 96-well plate static model based on the Calgary system and allowing the growth of a *P. aeruginosa in vitro* biofilm on lid pegs but in this case, the bottom of the microplate wells were coated with lung epithelial cells A549 (see Figure 25). Among their observations, we can mention a higher eukaryotic metabolism and IL-8 release induced by the presence of biofilms, by comparison with planktonic bacteria and control conditions (no bacteria) (Bowler *et al.*, 2014).

Figure 25



Schematic diagram of a 96-well plate and peg-lid set-up (Bowler *et al.*, 2014). The bulk of the biofilms does not contact the A549 cells. However dispersion of the biofilms occurs through the release of planktonic bacteria as well as released pieces of the biofilm.



IL-8 release from A549 cells as measured by ELISA after co-culture with biofilm and planktonic bacteria (Bowler *et al.*, 2014). Error bars indicate standard deviation of four biological repeats.

3.5. Anti-biofilm strategies

Adhesion inhibitors

Sortases produced by Gram positive bacteria, including *S. pneumoniae*, are membrane enzymes catalyzing covalent anchoring of surface proteins such as adhesins to peptidoglycans. Therefore, sortases inhibitors such as methane-thiosulfate or mercurial p-hydroxy-mercuribenzoic acid targeting sortase A, are considered as good candidates for preventing biofilm adhesion and development. They work by blocking the Cys184 in the active site of the enzyme (Chen and Wen, 2011). In addition to this type of preventing molecules, other technologies such as catheter coating with nanoparticules of yttrium fluoride (YF3) or MgF_2 have shown good prophylactic activity against development of *S. aureus* and *E. coli* biofilms (Lellouche *et al.*, 2012). Fluoride possesses anti-bacterial activity and this may partly be linked to their ability to alter prokaryote membrane integrity (Lellouche *et al.*, 2012). Fluoride antiglycolytic properties, interfering with the bacterial metabolic function and proliferation, have also been reported for different bacterial species, including buccal streptococci (Bunick and Kashket, 1981; Qin *et al.*, 2006).

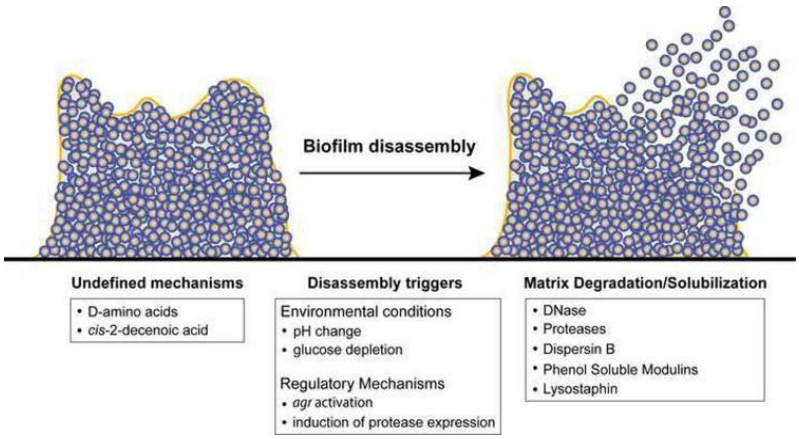
Quorum sensing inhibitors

These inhibitors are used to inhibit biofilm-mediated colonization by targeting its structure during the maturation phase (Chen and Wen, 2011). They can act by inhibiting (i) autoinducers generation (e.g. anhydroribosyl-L-homocystein and derivates inhibits LuxS), (ii) signal molecules or induce their degeneration with enzymes or (iii) the signal receptor and transduction with analogues of the signal molecule (Chen and Wen, 2011; Roy *et al.*, 2011).

Biofilm disassembly inducers

These compounds are possible candidates for disturbing the biofilm structure. Disassembly is defined as a natural multiple step process occurring in mature biofilms and responsible of (i) the degradation of the extracellular matrix with release of bacterial cells and (ii) physiological changes in bacteria that prepare them for living conditions outside the biofilm (Boles and Horswill, 2011). This process plays an important role in tissue colonization occurring in biofilm-associated infections. Biofilm disassembly is already well described for *S. aureus* in which environmental conditions and regulatory mechanisms are playing the role of disassembly inducers and enzymes (e.g. DNases, proteases) the role of effectors to proceed to matrix degradation/solubilization such as illustrated in Figure 26 (Boles and Horswill, 2011).

Figure 26



Model of known Staphylococcal biofilm disassembly mechanisms (Boles and Horswill, 2011)

In addition to mechanisms illustrated here above, it has also been reported that bacteria living within the matrix may secrete disassembly self-produced factors. D-amino-acids (e.g. D-Tyr, D-Leu, D-Trp, D-Met incorporated in the peptidoglycan layer and causing the release of amyloid fibers that link cells in the biofilm together) or polyamines (e .g. norspermidine interacting with matrix polysaccharides) are some of these compounds and are presented as interesting strategies to deal with biofilms (Kolodkin-Gal *et al.*, 2010; Kolodkin-Gal *et al.*, 2012).

Matrix thickness targeting

Some pneumococcal hydrolases playing a role of virulence factor (e.g. autolysin A, N-acetylmuramoyl-l-alanine amidase, lysozyme LytC) also have a matrix disintegration capacity observed in early maturity stages (14-16h)(Domenech *et al.*, 2011). Similar activity on matrix is obtained with DNases treatment on young and old biofilms (Hall-Stoodley *et al.*, 2008).

Another type of matrix targeting is the Phage therapy. Coating of medical devices with a mix "bacteria and bacteriophages" is described as a good prophylactic option to avoid early biofilm development at their surface. This has essentially been observed for biofilms made by Gram negative bacteria. For example, in 2012, Liao and colleagues published results showing that when urinary catheters were totally covered by an *E.coli* non-pathogenic biofilm and pre-treated with lytic phages before infection with *P. aeruginosa*, this last pathogen was not able to develop on these structures. Bacteriophages were used to enhance the persistence of benign *E.coli* used as second coating agent (Liao *et al.*, 2012). Bacteriophages have also been reported as possessing themselves a lytic activity on *P. aeruginosa*, in addition to a deleterious effect on their physiological function (e.g. mobility) (Hosseinidoust *et al.*, 2013).

Pharmacologic treatment seems to be also a possible anti-matrix thickness strategy. Indeed, subinhibitory concentrations of macrolides (e.g. azithromycin, against *H. influenzae*, and clarithromycin, against *P. aeruginosa*) inhibit biofilm formation and target established biofilms without killing cells through an actually unknown mechanism (Starner *et al.*, 2008; Yasuda *et al.*, 1993). Concerning non-antibiotic drugs, ibuprofen, a non-steroidal anti-inflammatory drug, shows decreasing and inhibiting activities on biofilm formation in young *S. pneumoniae in vitro* models at concentrations of $\geq$0.25mg/L and 1mg/L, respectively (del Prado *et al.*, 2010).

This shows that, such as for efflux-mediated resistance, non-antibiotic drugs may influence antimicrobial activity on biofilms. Because of the high implication of this concept in clinical practice for polymedicated patients and the importance of a better understanding of the underlying mechanisms, the following review article bundles actual knowledge concerning modulation of the antibiotic activity on respiratory pathogens by non-antibiotic drugs.

Aims

The World Health Organization defines Chronic Bronchitis or Chronic Obstructive Pulmonary Disease (COPD) as one of the five major causes of death worldwide (Minino and Murphy, 2012). Amongst severity risk factors, bacterial acute exacerbations episodes (AECB) take a predominant place, partly because of their recurrent component that implicates repeated administration of antibiotics to the patients. Frequent use of these drugs, in addition to inner bacterial resistance mechanisms and particular life modes such as growth within biofilms, increases therapeutic fails and the selection of resistance strains. *Streptococcus pneumoniae*, a Gram positive bacterium, is one of the most frequent pathogens involved in exacerbations episodes (Eller *et al.*, 1998). In that context, the aim of this thesis built on the basis of translational research, was to explore some pneumococcal mechanisms reducing the antibiotic activity and, to the extent possible, to establish links between experimental *in vitro* results and patients physio-pathological characteristics observed and described in clinics. For that purpose, the project was divided in three main paths of investigations.

First of all, from an epidemiologic point of view, we collaborated with five Belgian hospitals during three years to collect *S. pneumoniae* clinical isolates and medical data from AECB patients. Patients were characterized for several demographic parameters and for their comorbidities, length of hospitalization, smoking habit and medications, in order to be able to compare the characteristics of our AECB patients population with what is actually described in the literature for patients suffering from the same disease. The whole collection of clinical strains was characterized for the susceptibility to antibiotics, the prevalence of efflux, the serotype and the ability to produce biofilm.

This latter aspect constitutes the transition between the clinical and experimental parts of our work. Indeed, in parallel to this analysis of the problem of antibiotic resistance observed in clinical practice, a second main aim of this work was to develop *in vitro* models of young to very mature biofilms mimicking primer or secondary infections and made by reference strains or clinical isolates collected in the sputum of COPD patients. These models were used to characterize the pneumococcal biofilm production overtime and to study and compare, through pharmacodynamic studies, the antibiotic activity towards biofilm thickness and the viability of pneumococcal cells living within the biofilm matrix.

Then, in order to be as close as possible to the clinical aim, we studied the impact of pneumococcal incubation with non-antibiotic drugs, such as bronchodilators and benzodiazepines, or nicotine at concentrations relevant of those found in COPD patients airways, on the *in vitro* development of biofilms and on the antibiotic activity towards these structures.

Finally, additional experiments of pneumococcal characterization and set-up of new assays were made to increase our general knowledge about *S. pneumoniae* behavior and the range of techniques of our laboratory.

Results

«Tout va bien aller. Peut-être pas aujourd'hui, mais éventuellement...»

1. Epidemiological study

During the last ten years, our laboratory already studied the problem of antibiotic resistance in *S. pneumoniae* strains isolated from patients suffering from community-acquired pneumonia (CAP), a respiratory infection mainly affecting infants and the elderly. This previous study was mainly focused on the susceptibility to antibiotics, on efflux and on the serotypes, in relation to pneumococcal vaccination (Lismond *et al.*, 2012).

Upon discussion with clinicians, we thought that it could be interesting to extend our investigations to pneumococcal acute exacerbations episodes occurring in patients suffering from chronic bronchitis, a chronic respiratory disease. Indeed, because of the infections recurrence in this population, patients are receiving more frequently antibiotics than for an acute infection, such as CAP. Therefore, we wanted to check if this higher antibiotic use leads to an increase of Pneumococci resistance in Belgium.

Moreover, because bacterial persistence within biofilms is considered to be involved in up to 80% of chronic infections, we investigated this particular bacterial life mode in these clinical isolates and tried to establish some correlations with the other studied parameters, such as described here below.

AUTHORS' CONTRIBUTIONS:

1. Conceived and designed experiments: NMV, PMT, FVB;
2. Performed experiments: NMV, YDI, JV;
3. Analyzed data: NMV, PMT, FVB;
4. Contributed to medical data acquisition and strains: NMV, HRV, IP, JC, NC, AB, KVV, HF, RV, GL, PJ, J-P dO, YV, FV
5. Wrote the paper: NMV, PMT, FVB.

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Characterisation of a collection of *Streptococcus pneumoniae* isolates from patients suffering from acute exacerbations of chronic bronchitis: In vitro susceptibility to antibiotics and biofilm formation in relation to antibiotic efflux and serotypes/serogroups

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ABSTRACT

The correlation between *Streptococcus pneumoniae* serotypes, biofilm production, antibiotic susceptibility and drug efflux in isolates from patients suffering from acute exacerbations of chronic bronchitis (AECB) remains largely unexplored. Using 101 isolates collected from AECB patients for whom partial ($n = 51$) or full ($n = 50$) medical details were available, we determined serotypes (ST)/serogroups (SG) (Quellung reaction), antibiotic susceptibility patterns [MIC (microdilution) using EUCAST and CLSI criteria] and ability to produce biofilm in vitro (10-day model; crystal violet staining). The majority of patients were 55–75 years old and <5% were vaccinated against *S. pneumoniae*. Moreover, 54% showed high severity scores (GOLD 3–4), and comorbidities were frequent including hypertension (60%), cancer (24%) and diabetes (20%). Alcohol and/or tobacco dependence was >30%. Isolates of SG6-11-15-23, known for large biofilm production and causing chronic infections, were the most prevalent (>15% each), but other isolates also produced biofilm (SG9-18-22-27 and ST8-20 being most productive), except SG7, SG29 and ST5 (<2% of isolates each). Resistance (EUCAST breakpoints) was 8–13% for amoxicillin and cefuroxime, 35–39% for macrolides, 2–8% for fluoroquinolones and 2% for telithromycin. ST19A isolates showed resistance to all antibiotics, ST14 to all except moxifloxacin, and SG9 and SG19 to all except telithromycin, moxifloxacin and ceftriaxone (SG19 only). Solithromycin and telithromycin MICs were similar. No correlation was observed between biofilm production and MIC or efflux (macrolides, fluoroquinolones). *S. pneumoniae* serotyping may improve AECB treatment by avoiding antibiotics with predictable low activity, but it is not predictive of biofilm production.

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1. Introduction

Chronic obstructive pulmonary disease (COPD) remains one of the major causes of morbidity and mortality worldwide, occupying the fourth place for death since 2000 and predicted to reach third place in 2020 [1,2]. At increasingly closer intervals, COPD patients suffer from acute exacerbations of chronic bronchitis (AECB), which contribute to alteration of their respiratory function. These episodes are characterised by increased dyspnoea, coughing and sputum production, being evidence of infection of the airways [3]. Bacterial pathogens are found in 50–80% of cases of AECB [4], with *Streptococcus pneumoniae* being one of the dominant species [1,4]. Recurrence of infections associated with bacterial persistence results in frequent antibiotic courses. This favours the emergence of multiresistance of *S. pneumoniae* [5] through a variety of non-mutually exclusive mechanisms such as alterations in the antibiotic targets for β-lactams and macrolides as well as overexpression of efflux pumps for macrolides and fluoroquinolones [6]. Biofilm formation also favours the persistence of *S. pneumoniae* in the airways [7]. Up to 80% of chronic infections involve pneumococcal growth and survival within biofilms [8,9], in direct relation to the ability of this organism to colonise the nasopharynx [10], which may be dependent on its serotype (ST)/serogroup (SG) [11]. Whilst more than 90 distinct STs have been described for *S. pneumoniae* [12], few studies have attempted to examine what correlations exist between ST and/or SG and the ability to form thick biofilms in clinical isolates from patients with COPD [1]. Moreover, none of these studies have extended the correlations to key properties of the isolates such as their susceptibility to commonly recommended antibiotics and the expression of efflux transporters. Since efflux is critical in other bacteria for liberating quorum-sensing signalling molecules involved in biofilm formation [13], we also investigated the relationship between the ability of *S. pneumoniae* to form biofilm and the presence of phenotypic efflux for macrolides and ciprofloxacin.

In the present report, we show (i) that the susceptibility of *S. pneumoniae* isolates from COPD patients to β-lactams and fluoroquinolones is lower than that seen for patients with a confirmed diagnosis of bacterial community-acquired pneumonia (CAP) [14], (ii) that most of these isolates produce large amounts of biofilm irrespective of their ST/SG and (iii) that there is no correlation between the ability for biofilm formation in vitro and susceptibility to antibiotics or efflux towards macrolides or fluoroquinolones amongst the isolates investigated.

2. Materials and methods

2.1. General outline of the clinical study, patient selection and medical data acquisition

A first series of isolates consisted of 48 non-duplicate *S. pneumoniae* strains obtained between March 2006 and December 2008 from patients with a declared diagnosis of AECB and was assembled at the Belgian Scientific Institute of Public Health (Brussels, Belgium). Samples from this collection were equally distributed between the Belgian provinces in relation to their population. The second series of isolates consisted of 53 non-duplicate strains obtained in a prospective fashion between November 2010 and May 2013. For this purpose, five hospitals (one teaching and four non-teaching) were contacted and asked to enrol patients with a suspicion of AECB whether self-referred or referred by a general practitioner. Patients were enrolled upon obtaining a sample of sputum from the lower respiratory tract fulfilling the microbiological interpretive criteria of an acceptable specimen for culture [abundance of white blood cells (WBCs), few epithelial cells at low-power magnification, and ≥10–25 WBCs with no epithelial cells under 1000× magnification]. Only patients with samples yielding a positive culture for *S. pneumoniae* and with a confirmed diagnosis of AECB based on Anthonisen's criteria [3] were retained. For 50 of these patients, the whole medical data could be collected and was anonymised. Patients were stratified based on the severity scores (1–4 classification of the 2013 edition of the Global Initiative for Chronic Obstructive Lung Disease [GOLD] report [15]), sex, age, length of hospitalisation, geographical location, co-morbidities, smoking habit and therapeutic treatment at admission. Smoking habits were obtained from the patient's declaration. Tobacco usage was converted into 'pack × year' units by multiplying the number of packs smoked per day by the number of years as a smoker (using a threshold of >20 for increased risk of tobacco-related cancer [16]).

2.2. Bacterial strains and growth conditions

After identification in each clinical laboratory, strains were stored at −80 °C for transfer to a central laboratory until used for our experiments. Confirmation of identification was made by growth inhibition by optochin (Oxoid Ltd., Basingstoke, UK), and serotyping was performed as previously described [17]. *Streptococcus pneumoniae* ATCC 49619 strain (capsulated ST19F [18]) was used for quality control in each set of experiments. All strains were grown on Mueller–Hinton blood agar plates supplemented with 5% defibrinated horse blood incubated at 37 °C in a 5% CO_2 atmosphere.

2.3. Susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by microdilution following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [19]. MICs were read after 18–24 h of incubation at 37 °C. To improve accuracy, concentrations at half a value of each standard geometric progression were used as previously described [14] over the whole concentration range investigated. MICs were categorised as susceptible, intermediate or resistant according to the CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretive breakpoints [19,20].

2.4. Assessment of efflux phenotypes

The efflux resistance phenotype to macrolides was determined by examining the dissociation of susceptibilities between clarithromycin and clindamycin (substrate and non-substrate of the macrolide efflux transporters, respectively [14]). Efflux of fluoroquinolones was detected by a decrease in the MIC upon addition of reserpine (10 mg/L), an inhibitor of both PatA/B and PmrA fluoroquinolone efflux transporters in *S. pneumoniae* [14].

2.5. In vitro development of biofilms and determination of biofilm mass

Ninety-six well plates (European cat. no. 734-2327; VWR, Radnor, PA) were used as the support for biofilm growth. In each well, 25 μL of bacterial culture [optical density at 620 nm (OD_{620}) = 0.1] were added to 175 μL of cation-adjusted Mueller–Hinton broth supplemented with 5% lysed horse blood (Oxoid Ltd.) and 2% glucose as previously described [18]. Biofilm development was obtained by incubation for 2–10 days at 37 °C in a 5% CO_2 atmosphere with medium replacement every 48 h. Biofilms examined after 2 days or 10 days are referred to as young and mature biofilms, respectively. Biofilm mass was evaluated by staining with crystal violet followed by measuring the absorbance exactly as previously described [18]. Each isolate was tested twice at different dates, with each assay using three to six measures. The mean coefficient of

variation of the assay was 12.3% (extremes, 0.01–33.1). Data of all determinations for each isolate were pooled, and STs belonging to a given SG were regrouped after observing no significant differences in their capacity to form biofilm.

2.6. Antibiotics and other products

Amoxicillin, cefuroxime and ceftriaxone were obtained as the corresponding branded product for human parenteral use complying with the prescriptions of the European Pharmacopoeia (>90% purity) and distributed for clinical use in Belgium as, respectively, Clamoxyl® and Zinacef® by GlaxoSmithKline s.a./n.v. (Genval, Belgium) and Rocephin® by Roche s.a./n.v. (Brussels, Belgium). Clindamycin hydrochloride (potency 92.1%) was obtained from Sigma–Aldrich (St Louis, MO). Clarithromycin and azithromycin (potencies 100%) were from Teva Pharmaceutical Industries (Petah Tikva, Israel); telithromycin (potency 100%) and levofloxacin hemihydrate (potency 97.5%) were from Sanofi-Aventis Deutschland GmbH (Frankfurt, Germany); solithromycin (potency 100%) was from Cempra Pharmaceuticals (Chapel Hill, NC); and moxifloxacin chlorhydrate (potency 90.9%) was from Bayer AG (Leverkusen, Germany). Reserpine was obtained from Fluka (Buchs, Switzerland). All other products were obtained from Sigma–Aldrich or E. Merck AG (Darmstadt, Germany).

2.7. Statistical analyses

Unpaired *t*-test, one-way analysis of variance (ANOVA) and contingency tables were made with GraphPad Prism® 4.02 and GraphPad InStat® 3.10 (GraphPad Software Inc., San Diego, CA) and recursive partitioning analyses with JMP® 10.0.2 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Main characteristics of patients

Table 1 shows the demographic characteristics of the whole patient population. Most patients were 55–75 years of age. For samples prospectively collected, two hospitals provided a number of samples markedly above the average (in proportion to their bed capacity) owing to their location in or proximity to industrial areas. Most of these patients were living at home prior to hospitalisation and were smokers (more than three-quarters currently active or former deep smokers). Most patients remained hospitalised after reporting. The severity of their disease was equally distributed between low (1 or 2) and high (3 or 4) GOLD scores. Co-morbidity percentages ranged from 20% to 30% for diabetes, cancer and alcoholism, and to 60% for arterial hypertension.

3.2. Correlations between demographic, clinical and pharmaceutical parameters

Associations between demographic factors, severity of disease, co-morbidities and drug usage were examined in 50 patients for whom full medical records were available. Table 2 shows that the length of hospitalisation was clearly correlated with the severity of disease and with tobacco dependence. Older patients had a more severe obstructive syndrome and were more often hypertensive, were poorly vaccinated and showed less incidence of alcoholism.

3.3. Serotype/serogroup analyses

Serotyping was performed on all isolates ($n = 101$). Fig. 1A shows the distribution of STs/SGs amongst the two successive series of isolates (2006–2008 and 2010–2013) stratified by level of coverage (partial or total) with PCV7 [7-valent pneumococcal conjugate vaccine (Prevenar®; Wyeth)] and PPV-23 [23-valent pneumococcal polysaccharide vaccine (Pneumo23®; Sanofi-Pasteur MSD)] (the two vaccines in usage at the time during which most isolates were obtained), and for each of these groups by frequency. Whilst there were some changes in ST/SG frequencies between the two series of isolates, SG6 and SG23 were the most prevalent throughout. The second series of isolates also contained a large proportion of SG11 and SG15 strains. There was no marked heterogeneity in ST/SG between the contributing regions (based on patients' living place records; see Supplementary Fig. S1). Globally speaking, only 5% of patients hospitalised during the 2010–2013 period had been vaccinated but most isolates were actually from a ST/SG not fully

Table 1
Patients' demographic characteristics and environmental and medical conditions.

1. Whole population (2006–2008 and 2010–2013) ($n = 101$)

Age and no. enrolled	Mean ± S.D. (years)	<55 years	≥55 to <65 years	≥65 to <75 years	≥75 to <85 years	≥85 years
	67.2 ± 12.7	11 (10.9%)	33 (32.7%)	30 (29.7%)	23 (22.8%)	4 (4.0%)

2. Prospectively assembled population (2010–2013) ($n = 53$)

Hospital	A	B	C	D	E	Total
No. enrolled	8	8	3	20	14	53
% of capacity[a]	0.8	0.95	0.36	3.6	4.7	2.1 ± 1.94 (mean ± S.D.)

3. Patients from prospectively assembled population and with available medical data ($n = 50$)

General and environmental conditions	Sex (% M/F): 74/26	Living place (% home/nursing home/psychiatric institution): 88/4/8	Smoking habits[b] (% active/former/non-smoker/unknown): 56/28/6/10	
Disease severity	Hospitalisation (% yes/no): 80/20		GOLD score (% 1 or 2/3 or 4): 46/54	
Co-morbidities	Hypertension[c] (%): 60	Diabetes[d] (%): 20	Cancer[e] (%): 24	Alcoholism[f] (%): 30

S.D., standard deviation; GOLD, Global Initiative for Chronic Obstructive Lung Disease [15].
[a] No. of enrolled patients/no. of beds in the hospital × 100.
[b] According to patient's declaration.
[c] Systolic blood pressure >120 mmHg.
[d] Fasting glycaemia >1.26 g/L.
[e] Tissue biopsies and/or chest radiographs.
[f] According to patient's declaration, evidence at admission (inebriated condition) or presence of alcoholic cirrhosis.

Table 2
Associations between length of hospitalisation, age, co-morbidities and vaccine serotype coverage (variables #1) with disease severity (GOLD score 3 or 4), prolonged hospitalisation, age and tobacco addition (variables #2) in the population of patients with fully accessible medical records ($n = 50$). Associations were tested by means of 2×2 contingency tables (Fisher's exact two-tailed test). The table shows the odd ratios (with their 95% confidence interval) and appear in bold if the P-value is ≤ 0.05 (some associations with a P-value between 0.05 and 0.1 are shown in italic if considered potentially medically important).

Variable #1	Variable # 2			
	Disease severity[a]	Hospitalisation >10 days	Age >65 years	Tobacco addiction [b]
Hospitalisation >10 days	**2.987 (1.242–7.182), $P < 0.05$**		1.289 (0.571–2.91), ns	**3.201 (0.9928–10.322), $P < 0.05$**
Age >65 years	**2.963 (1.296–6.772), $P < 0.05$**	1.289 (0.571–2.91), ns		*0.408 (0.16–1.039), P = 0.0761*
High blood pressure[c]	0.641 (0.284–1.451), ns	0.835 (0.369–1.889), ns	**3.947 (1.688–9.233), $P < 0.01$**	*2.545 (0.9643–6.719), P = 0.07*
Alcoholism[d]	*0.424 (0.180–1.000), P = 0.0541*	1.128 (0.469–2.712), ns	**0.087 (0.031–0.246), $P < 0.0001$**	1.071 (0.389–2.952), ns
Cancer [e]	*2.7 (0.966–7.548), P = 0.06*	0.767 (0.292–2.013), ns	*2.700 (0.966–7.548), P = 0.06*	*0.353 (0.117–1.058), P = 0.07*
Vaccine coverage[f]	1.250 (0.416–3.758), ns	0.490 (0.146–1.649), ns	**0.185 (0.0549–0.625), $P < 0.01$**	1.071 (0.316–3.634), ns

[a] Global Initiative for Chronic Obstructive Lung Disease (GOLD) score 3 or 4 [15].
[b] >20 'pack × years'.
[c] Systolic blood pressure >120 mmHg.
[d] According to patient declarations, evidence at admission (inebriated condition) or presence of alcoholic cirrhosis.
[e] Tissue biopsies and/or chest radiographs.
[f] Vaccine PCV13 [13-valent pneumococcal conjugate vaccine (Prevenar 13®; Wyeth)] covers serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F; and vaccine PPV-23 [23-valent pneumococcal polysaccharide vaccine (Pneumo23®; Sanofi-Pasteur MSD)] covers serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.

covered by the two vaccines examined (adding the STs covered by the PCV13 did not much change this pattern). Fig. 1B shows the STs/SGs of all isolates when stratified as a function of their ability reported in the literature of being (i) high biofilm producers and causing chronic infections [1,9], (ii) low biofilm producers and causing acute infections [9,14,21] or (iii) with undescribed ability for these characteristics. Approximately one-half of the isolates were found in the first group.

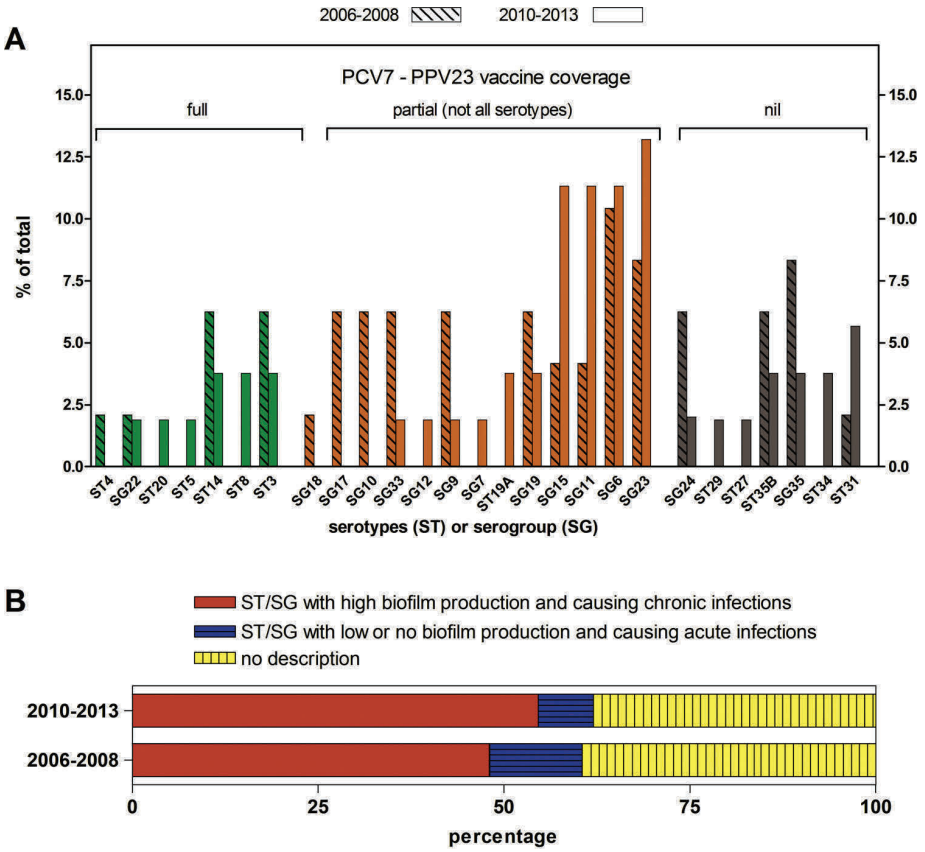

Fig. 1. (A) Distribution of isolates as a function of their period of collection (shaded blocks, 2006–2008; open blocks, 2010–2013) and serotype (ST)/serogroup (SG) and grouped following the vaccine coverage. PCV7, 7-valent pneumococcal conjugate vaccine (Prevenar®; Wyeth) covers ST4, 6B, 9V, 14, 18C, 19F and 23F (discontinued in 2011); PCV13, 13-valent pneumococcal conjugate vaccine (Prevenar 13®; Wyeth) covers ST1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F; and PPV-23, 23-valent pneumococcal polysaccharide vaccine (Pneumo23®; Sanofi-Pasteur MSD) covers ST1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. (B) Percentages of STs/SGs belonging to high biofilm producers and/or causing chronic infections (red bars; ST/SG 6, 9, 11, 15, 23, 33 and 35B), low biofilm producers and/or causing acute infections (blue bars; ST/SG 3, 14 and 19A) or with undescribed characteristics (all others; yellow bars; see text for references). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

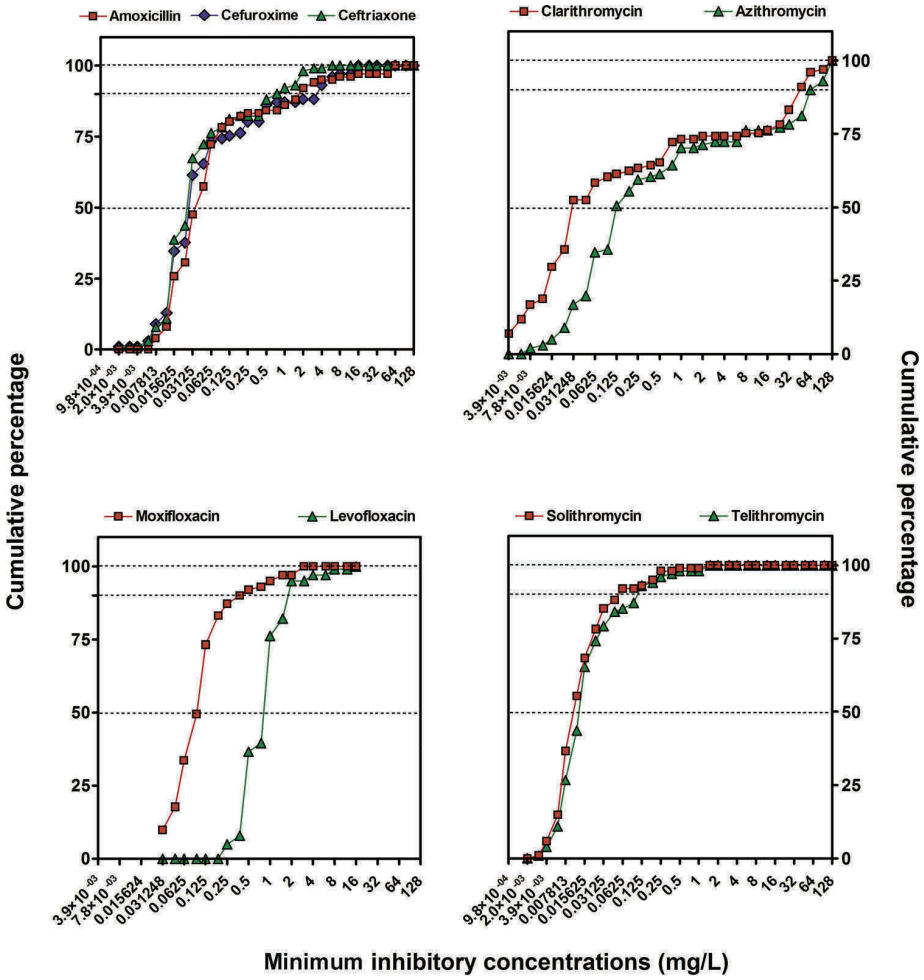

Fig. 2. Minimum inhibitory concentration (MIC) distributions (cumulative percentages) of β-lactams (amoxicillin, cefuroxime and ceftriaxone; upper left panel), macrolides (clarithromycin and azithromycin; upper right panel), fluoroquinolones (moxifloxacin and levofloxacin; lower left panel) and ketolides (telithromycin and solithromycin; lower right panel) for 101 non-duplicate *Streptococcus pneumoniae* isolates from chronic obstructive pulmonary disease patients. The horizontal dotted lines are drawn at values corresponding to the MIC_{50}, MIC_{90} and MIC_{100} (MICs required to inhibit 50%, 90% and 100% of the isolates, respectively).

3.4. Characterisation of the in vitro susceptibility to antibiotics and correlation with the severity of disease and serotypes/serogroups

All of the strains were characterised for their susceptibility to antibiotics focusing on (i) drugs commonly recommended for the ambulatory treatment of bacterial exacerbations of chronic bronchitis in Belgium at the time of the study (amoxicillin, cefuroxime [administered as its axetil prodrug] and moxifloxacin [22]) and (ii) ceftriaxone (an often prescribed β-lactam in hospitals), clarithromycin and azithromycin (often used in combination with a β-lactam) and levofloxacin (as an alternative antipneumococcal fluoroquinolone). We also added two ketolides [telithromycin (approved in Europe for the treatment of exacerbations of chronic bronchitis) and solithromycin (still investigational)] because of their reported good activity against *S. pneumoniae* resistant to macrolides [23], and tested clindamycin and ciprofloxacin for efflux diagnostic purposes. Cumulative MIC distributions are shown in Fig. 2 (see also Supplementary Fig. S2). MIC_{50} and MIC_{90} values (MIC required to inhibit 50% and 90% of the isolates, respectively) and analysis of the MIC profiles according both to EUCAST and CLSI interpretive criteria are presented in Table 3 for all strains and for each collection individually. There were no significant differences in susceptibilities between the two strain collections. Moreover, largely similar distributions were observed for all three β-lactams except in the zones corresponding to their clinical breakpoints, with 6–8% of all isolates falling into the intermediate category for the three drugs and 8–13% in the fully resistant category for amoxicillin and cefuroxime (using, for the latter, the interpretive criteria set for its oral form), but only 1% for ceftriaxone, based on EUCAST interpretive criteria (using CLSI interpretive criteria essentially resulted in having no or only one isolate in the intermediate category). For macrolides, isolates within the first half of the cumulative distribution were globally more susceptible to clarithromycin than azithromycin. The difference, however, largely disappeared for isolates with higher MICs. Resistance rates to clarithromycin and azithromycin reached 28% and 39% based on EUCAST interpretive criteria, and 27% and 29% based on CLSI interpretive criteria. Isolates categorised as intermediate were rare (<10%). The cumulative clindamycin MIC distribution was essentially similar to that of azithromycin, indicating that most of the strains categorised as resistant to this macrolide were of the MLS_B type. For fluoroquinolones, moxifloxacin was systematically more active than levofloxacin, but due to the lower breakpoint set by EUCAST for moxifloxacin, more strains (8%) were categorised as being resistant compared with levofloxacin (3%). No meaningful difference with respect to susceptibility was seen if using CLSI breakpoints. For ciprofloxacin, the majority of isolates had a MIC in the intermediate

Table 3
MIC_{50} and MIC_{90} values and percentages of non-susceptible (intermediate and resistant) isolates according to European Committee for Antibiotic Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) interpretive criteria.

Antibiotic	Strains collection	MIC (mg/L)		% Susceptibility according to:			
		MIC_{50}	MIC_{90}	EUCAST [20]		CLSI [19]	
				Breakpoint (S/R) (mg/L)	% I/R	Breakpoint (S/R) (mg/L)	% I/R
Amoxicillin	Global	0.046875	2	≤0.5/>2	8/8	≤2/≥8	1/4
	2006–2008	0.02344	1.5		10/4		0/0
	2010–2013	0.06250	4		6/11		2/9.4
Cefuroxime	Global	0.03125	4	≤0.25/>0.5 [a]	6/13	≤1/≥4[a]	1/7
	2006–2008	0.03125	4		6.3/14.6		2/8.4
	2010–2013	0.01563	4		5.7/11.3		0/5.7
Ceftriaxone	Global	0.03125	0.75	≤0.5/>2	8/1	≤2/≥4	0/1
	2006–2008	0.03125	2		10.4/2		0/2
	2010–2013	0.01563	0.5		5.7/0		0/0
Clarithromycin	Global	0.03125	48	≤0.25/>0.5	1/27.7	≤0.25/≥1	7.9/26.7
	2006–2008	0.03125	48		0/31.2		0/30.2
	2010–2013	0.03125	64		2/24.5		15.1/22.7
Azithromycin	Global	0.125	64	≤0.25/>0.5	1/38.6	≤0.5/≥2	6/28.7
	2006–2008	0.125	64		0/31.2		0/31.2
	2010–2013	0.250	128		0/39.6		11.3/26.4
Clindamycin[b]	Global	0.0625	32	≤0.5/>0.5	0/35.7	NA	NA
	2006–2008	0.046875	16		0/27		NA
	2010–2013	0.0625	48		0/43.4		NA
Telithromycin	Global	0.015625	0.125	≤0.25/>0.5	1/2	≤1/≥4	0/0
	2006–2008	0.015625	0.0625		0/2		0/0
	2010–2013	0.015625	0.125		2/2		0/0
Solithromycin	Global	0.01172	0.0625	NA	NA	NA	NA
	2006–2008	0.01172	0.046875		NA		NA
	2010–2013	0.00781	0.0625		NA		NA
Moxifloxacin	Global	0.125	0.375	≤0.5/>0.5	0/8	≤1/≥4	3/0
	2006–2008	0.125	0.5		0/6.3		2/0
	2010–2013	0.09375	0.375		0/9.4		4/0
Levofloxacin	Global	1	2	≤2/>2	0/3	≤2/≥8	2/1
	2006–2008	1	2		0/2		0/0
	2010–2013	0.75	1.5		0/4		3.8/2
Ciprofloxacin[b]	Global	1	4	≤0.125/>2	82.2/13.8	NA	NA
	2006–2008	1	4		87.5/10.4		NA
	2010–2013	1	4		77.4/17		NA

MIC, minimum inhibitory concentration; $MIC_{50/90}$, MIC required to inhibit 50% and 90% of the isolates, respectively; S, susceptible; R, resistant; I, intermediate; NA, not applicable (no breakpoint defined).
[a] Oral form (cefuroxime axetil).
[b] Not recommended for clinical use but tested here for epidemiological purposes.

category of EUCAST (no breakpoint set for CLSI). We examined the occurrence of efflux for ciprofloxacin by addition of reserpine. As illustrated in Fig. 3, there was a shift of the whole population towards lower MICs, with 38% and 35% of the isolates showing a 1 or $\geq 2 \log_2$ dilution changes, respectively. Lastly, the cumulative MIC distributions of telithromycin and solithromycin were very similar, with few (EUCAST) or no isolate (CLSI) categorised as resistant (breakpoints for solithromycin have not yet been set).

We then examined the correlation between the severity of disease and resistance of the isolates to amoxicillin and cefuroxime by stratifying patients by low (1 or 2) and high (3 or 4) GOLD scores, respectively, and performing a recursive partitioning analysis versus the MICs of their isolates. Whilst this allowed determination of a best MIC split value at 4 mg/L for amoxicillin and at 1 mg/L for cefuroxime, this was not statistically significant (LogWorth values = 0.18 and 0.17, respectively, corresponding to *P*-values of 0.66 and 0.68). This was further confirmed by 2×2 contingency table analysis (*P*-values of 0.18 and 0.31).

Fig. 4 shows the susceptibilities of the strains grouped by ST/SG for each of the antibiotics tested in Fig. 2 and ranked by their mean MIC value (from highest to lowest) with the corresponding EUCAST and CLSI intermediate susceptibility zones. Although the ranking of STs/SGs varied between antibiotics, global trends emerged with ST14, ST19A, SG9, SG29 and SG15 having the highest mean MICs for β-lactams, ST19A, SG9, ST14, SG19 and SG 33 for macrolides, ST14, ST19A, SG19, SG9 and SG17 for both ketolides, and ST19A, SG33, ST4, ST5 and SG15 for moxifloxacin and levofloxacin, respectively. All ST19A isolates had a MIC above the EUCAST resistance breakpoint for amoxicillin, cefuroxime, clarithromycin, azithromycin, moxifloxacin and levofloxacin. Conversely, SG7 and ST8 isolates were fully susceptible to all antibiotics (see Supplementary Table S1 for a ranking of all isolates stratified by ST/SG, MICs and resistance pattern).

3.5. Biofilm production in relation to pneumococcal susceptibility to antibiotics (minimum inhibitory concentrations and occurrence of efflux)

No significant correlation (one-way ANOVA with Tukey's post-test) was seen between biofilm production (crystal violet staining) and antimicrobial susceptibility (MIC) for each of the antibiotics tested (see data in Supplementary Fig. S3). Likewise, there was

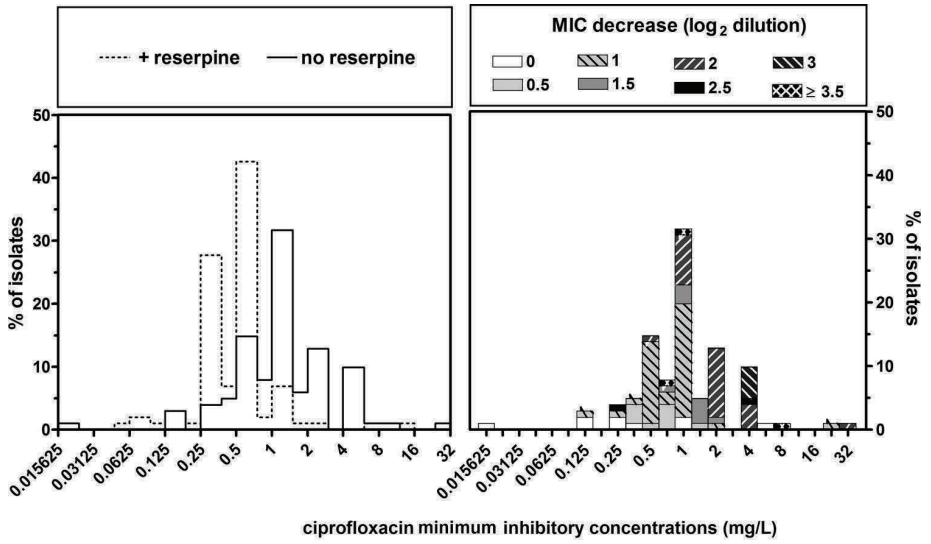

Fig. 3. Minimum inhibitory concentration (MIC) distribution of ciprofloxacin for all isolates ($n = 101$). Left: MIC distributions determined in the absence (control; solid line) or presence (dotted line) of 10 mg/L reserpine [statistical analysis, $P < 0.0001$ when comparing distributions in the absence and presence of reserpine by two-tailed paired tests; Wilcoxon signed-rank test (non-parametric) and t-test (parametric)]. Right: reduction of MIC (in blocks of 0.5 $\log_2$ dilutions from 0 to $\geq$3.5 $\log_2$ dilutions) after addition of 10 mg/L reserpine and plotted as a function of the MIC distribution of the isolates in the absence of reserpine.

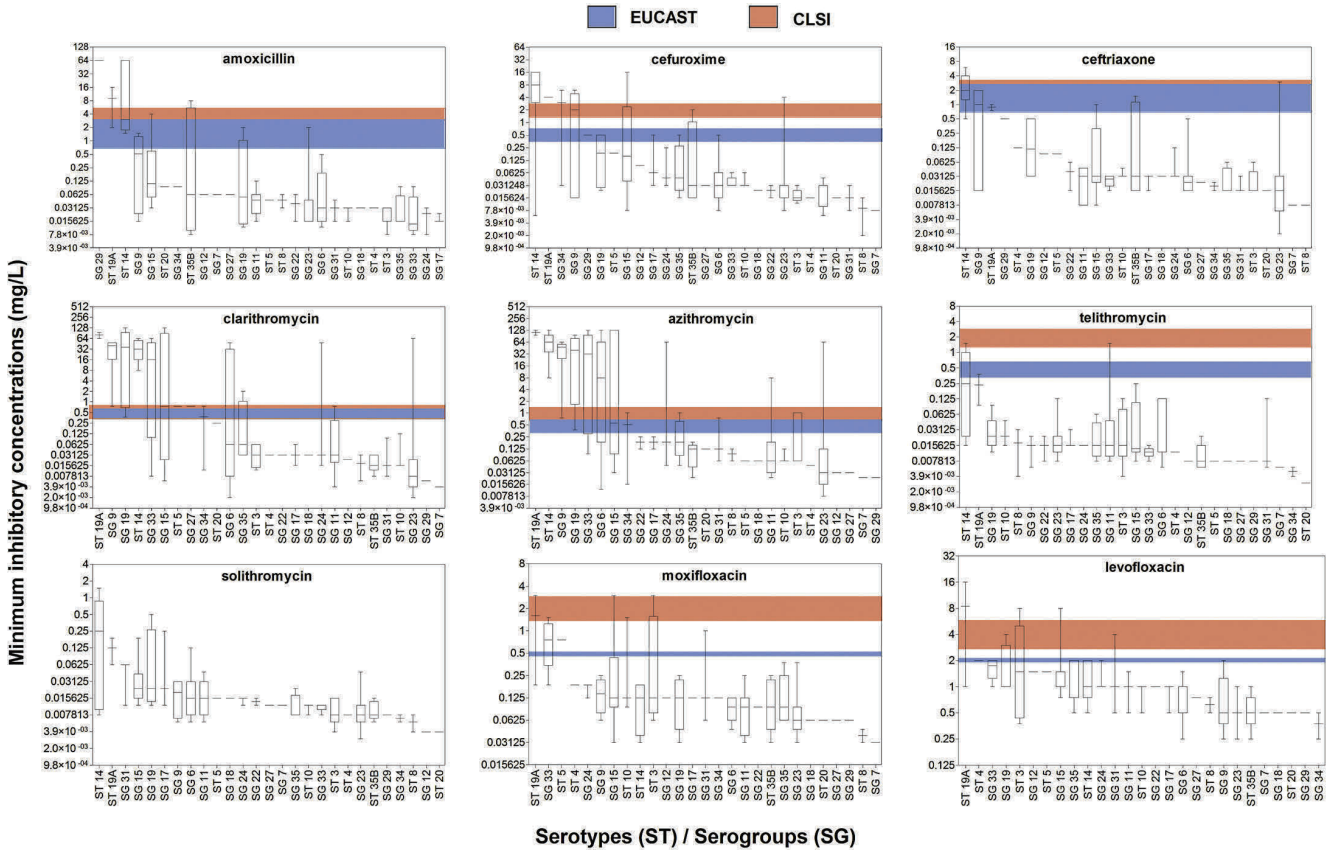

Fig. 4. Antibiotic susceptibility to β-lactams (amoxicillin, cefuroxime and ceftriaxone), macrolides (clarithromycin and azithromycin), ketolides (telithromycin and solithromycin) and fluoroquinolones (moxifloxacin and levofloxacin) for all isolates as a function of their serotype (ST)/serogroup (SG) ranked from less to more susceptible. Data are presented as box and whiskers plots giving the 25, 50 and 75 quartiles (boxes and horizontal line) of the minimum inhibitory concentration (MIC) distributions, with the lower and upper bars extending from the lowest to the highest MIC value observed. The blue and pink horizontal ribbons show the intermediate zones of clinical susceptibility according to the interpretive criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (from >S to >R; [20]) and the Clinical and Laboratory Standards Institute (CLSI) (from >S to <R [19]), respectively (see Table 3 for values; for clarithromycin, the intermediate zone is the same for EUCAST and CLSI; no clinical breakpoint has been set so far for solithromycin). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

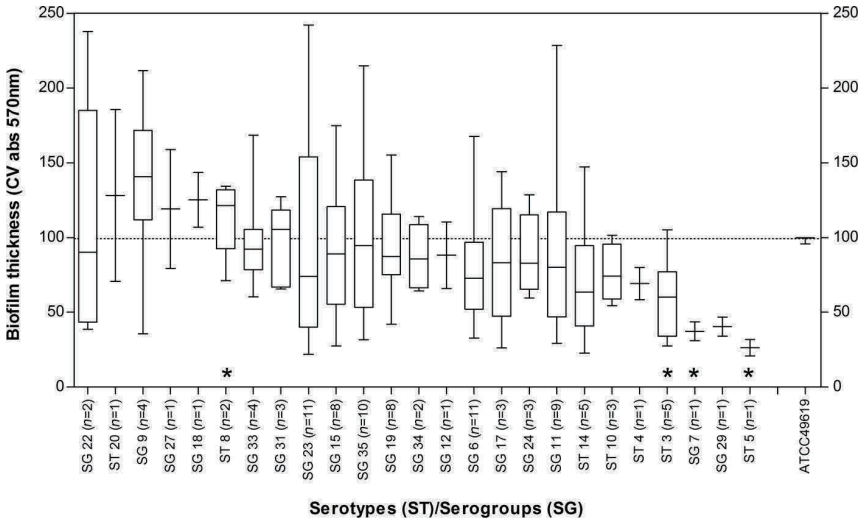

Fig. 5. Biofilm production after 10 days of culture for all clinical isolates as a function of their serotype (ST)/serogroup (SG) and ranked from the most to the least productive [using arithmetic means; the box and whiskers show the lowest, the 25 and 75 percentiles, the median and the highest value] (the number of isolates tested for each ST/SG is shown on the abscissa) and of the reference strain *Streptococcus pneumoniae* ATCC 49619 (ST19F). Each strain was tested twice with three to six measures each time. Strains with STs belonging to a single SG have been pooled (and marked as SG) after having observed no significant differences between these STs. The horizontal dotted line corresponds to the mean value for the reference strain. Strains marked ST correspond to isolates where there was only one serotype. STs/SGs reported in the literature [1,9] as causing acute infections are marked with an asterisk. CV, crystal violet.

no statistically significant correlation (unpaired *t*-test, Welch corrected) between biofilm production and expression of efflux for macrolides or ciprofloxacin (see data in Supplementary Fig. S4).

3.6. Characterisation of biofilm formation in relation to pneumococcal serotype/serogroup

Fig. 5 shows the amount of biomass observed at Day 10 for the reference strain *S. pneumoniae* ATCC 49619 (ST19F) and for the clinical isolates ranked by inverse amount of production and regrouped by ST/SG. Globally, all isolates, except SG7, SG29 and ST5, produced biofilm in a similar fashion to the reference strains ATCC 49619, with SG22, ST20, SG9, SG27, SG18 and ST8 being the most productive (SG9 and SG22 have been previously reported to be associated with chronic infections [1,9]). Conversely, SG7, SG29 and ST5 were the lowest producers in this collection, and these have been reported as poor producers with ST5 and SG7 claimed to be associated with acute infections [9].

4. Discussion

To the best of our knowledge, the present study is one of the first examining in a systematic fashion and correlating the STs/SGs, the resistance pattern and in vitro biofilm formation ability of *S. pneumoniae* isolates collected from COPD patients with a confirmed diagnosis of AECB. The number of patients and corresponding microbiological samples were limited due to the design of the study, which implied access to the medical history of the patients on the one hand, and the low rate of successful isolation of *S. pneumoniae* in this patient population on the other hand.

Within these limits, we first confirm and strengthen the close link existing between the severity of COPD and cardiovascular and diabetes pathologies already described in the literature [2,24]. A decreased ability to breathe reduces mobility, thereby favouring a sedentary lifestyle and weight gain. We next confirm that β-lactams, levofloxacin and moxifloxacin maintain useful activity against *S. pneumoniae* isolates from this population, although to a lesser extent than what we saw in a previous study for isolates obtained in Belgium from patients suffering from CAP during the 2006–2009 period [14]. Whilst the two patient populations cannot be directly compared, they nevertheless originate from the same small geographical area, suggesting that we deal, at least partially, with distinct bacterial populations. The lower susceptibility of isolates obtained from COPD patients probably reflects the large and prolonged use of antibiotics in this population before eventually reporting to the hospital (most patients suffering from CAP and included in our previous study had not received any antibiotic when enrolled [14]). Our findings, therefore, call for caution against the non-documented use of β-lactams [especially cefuroxime (given orally as cefuroxime axetil)] in COPD patients. For macrolides, the situation is even more critical as resistance patterns are appalling. Telithromycin, approved in Europe for the treatment of infections caused by β-lactam- and macrolide-resistant strains, maintains a very high level of activity, probably because of its very low use in Belgium owing to its non-inclusion as a recommended antibiotic for the treatment of AECB in local guidelines [22] (solithromycin is still an investigational drug). Of note is the larger prevalence of efflux for ciprofloxacin, especially if considering the proportion of isolates where a MIC change of $\geq 2 \log_2$ dilutions could be observed upon addition of reserpine. Whilst moxifloxacin and levofloxacin were not significantly affected, we know from previous studies that efflux of ciprofloxacin can herald similar changes in MICs for other fluoroquinolones proposed for the treatment of respiratory tract infections such as gemifloxacin and garenoxacin [25].

Analysis of the susceptibility pattern in relation to their ST/SG shows that some of them (ST19A, ST14, SG9 and SG9) have a high level of resistance to β-lactams and macrolides [and decreased susceptibility to ketolides (ST19A and ST14 only)] but not to moxifloxacin (except for ST19A). This is largely akin to our previous findings for isolates from patients suffering from CAP [14] as well as data from other countries in Europe [26] and the Far East [27,28]. Thus, determination of the prevalent STs/SGs in patients may help in fine-tuning therapy by avoiding the use of antibiotics known to be poorly effective. Determination of the nucleotide sequence of the α-helical region of the pneumococcal surface protein (PspA), also proposed as a predictor of multiresistance

[28], could not be examined in the context of the present study.

Turning our attention now to biofilm production, we see that most isolates obtained in this study were high producers (similar to the reference strain ATCC 49619 [ST19F] also known as a high producer [1,18]). In the present study, careful attention was paid to obtain data as reproducible as possible for biofilm production. Thus, by and large, this production appears to be a property shared by most isolates from COPD patients, suggesting that previous reports linking poor biofilm production by some of the strains studied here to more acute infections may need revisiting [9]. Conversely, we confirm that strains previously reported to be largely associated with acute infections, such as ST8 and ST3 [1], are indeed poor biofilm producers.

Lastly, we show no correlation between biofilm formation and intrinsic susceptibility or expression of macrolide or ciprofloxacin efflux in the isolates studied. This could explain why the determination of susceptibility by the reference methods (which use planktonic cells) may fail to truly predict the clinical outcome. Eradication of bacteria from biofilms, indeed, requires antibiotic concentrations to be maintained at values much larger than the breakpoint, especially if considering β-lactams and macrolides against mature biofilms [18]. The lack of correlation between biofilm production and ciprofloxacin efflux, which is in contrast with what is observed in Gram-negative bacteria, probably relates to differences in quorum-sensing signalling pathways and the secretion of the corresponding mediators [13,29,30].

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Competing interests: NMV is boursière of the Belgian Fonds pour la Recherche dans l'Industrie et l'Agriculture (FRIA); FVB is Maître de Recherches of the Belgian Fonds de la Recherche Scientifique (F.R.S.-FNRS); PMT and FVB have received research grants from Fonds de la Recherche Scientifique [grants 3.4530.12 and T.0134.13] and the Interuniversity Attraction Poles Programme initiated by the Belgian Science Policy Office [program IAP P7/28], grants-in-aid from Cempra Pharmaceuticals and Bayer AG, and consultancy fees from Bayer AG. All other authors declare no competing interests.

Ethical approval: The entire study protocol was submitted and approved by the Commission d'éthique facultaire of the Université catholique de Louvain (Brussels, Belgium) [unique Belgian no. 40320109783]. The ethical committee of the participating hospitals also gave their approval for the specific studies and access to medical files in the corresponding institutions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2014.05.016.

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Supplementary Table S1

Isolate ranking for each serotype (ST)/serogroup (SG) as a function of their minimum inhibitory concentration (MIC). STs/SGs are ranked from the least susceptible (small ranking value) to the most susceptible (high ranking value)

β-Lactams [a]		Macrolides [b]		Ketolides [c]		Fluoroquinolones [d]	
ST/SG	Ranking [e]	ST/SG	Ranking [e]	ST/SG	Ranking [e]	ST/SG	Ranking [e]
ST 14	5	ST 19A	2	ST 14	2	ST 19A	2
ST 19A	7	SG 9	5	ST 19A	4	SG 33	5
SG 9	10	ST 14	6	SG 19	8	ST 4	6
SG 29	10	SG 19	7	SG 9	13	ST 5	10
SG 15	23	SG 33	10	SG 17	15	SG 15	15
SG 19	23	SG 15	12	SG 15	18	ST 3	15
SG 12	24	SG 34	16	SG 22	20	SG 19	16
ST 5	28	SG 6	17	SG 11	21	SG 24	16
SG 34	30	ST 20	24	ST 10	21	SG 12	17
ST 35B	34	ST 5	24	SG 24	22	ST 14	19
SG 27	43	SG 22	24	SG 6	24	ST 10	22
SG 22	43	SG 35	24	SG 31	27	SG 9	26
SG 11	45	SG 17	26	SG 35	27	SG 31	26
ST 10	49	SG 24	29	SG 23	29	SG 35	29
SG 6	50	SG 27	33	ST 5	30	SG 17	29
SG 17	51	ST 3	34	ST 8	30	SG 11	30
ST 4	52	ST 35B	35	SG 18	32	SG 22	33
SG 33	52	SG 18	35	ST 3	32	SG 6	33
ST 20	53	ST 4	36	SG 33	33	ST 35B	41
SG 24	53	ST 8	37	SG 27	36	SG 34	42
SG 18	54	SG 11	38	ST 4	37	SG 27	42
SG 35	56	SG 31	38	SG 7	40	SG 23	42
SG 23	61	SG 12	44	ST 35B	41	ST 8	45
SG 7	63	ST 10	44	SG 12	44	SG 18	46
SG 31	65	SG 23	48	SG 29	46	ST 20	48
ST 3	66	SG 29	53	SG 34	50	SG 7	50

ST 8	68	SG 7	53	ST 20	54	SG 29	51

[a] Amoxicillin, cefuroxime and ceftriaxone.

[b] Clarithromycin and azithromycin.

[c] Telithromycin and solithromycin.

[d] Moxifloxacin and levofloxacin.

[e] Ranking values were calculated by adding, for drugs belonging to the same antibiotic class, the numbers of ranking positions (from 1 to 27) of each ST/SG, following their classification in Fig. 3 (from the least to the most susceptible). The five most susceptible and resistant STs/SGs are marked in colour. Similar colours indicate similarities between antibiotic classes.

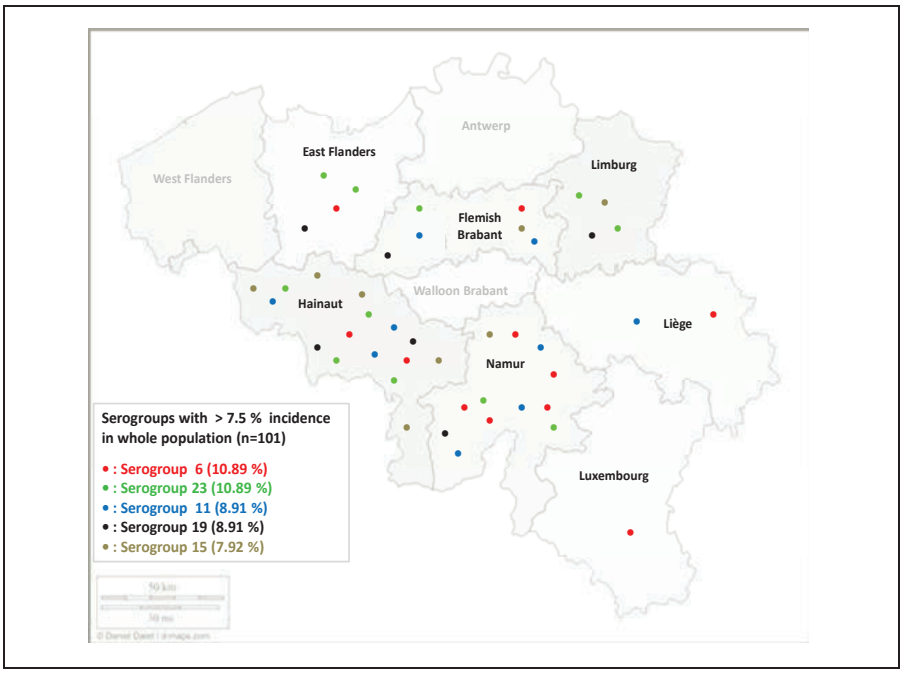
Supplementary Fig. S1. Distribution of serogroups with an incidence >7.5% in the whole population (*n* = 101) across the provinces where patients were living (provinces with no patients are labelled in grey).

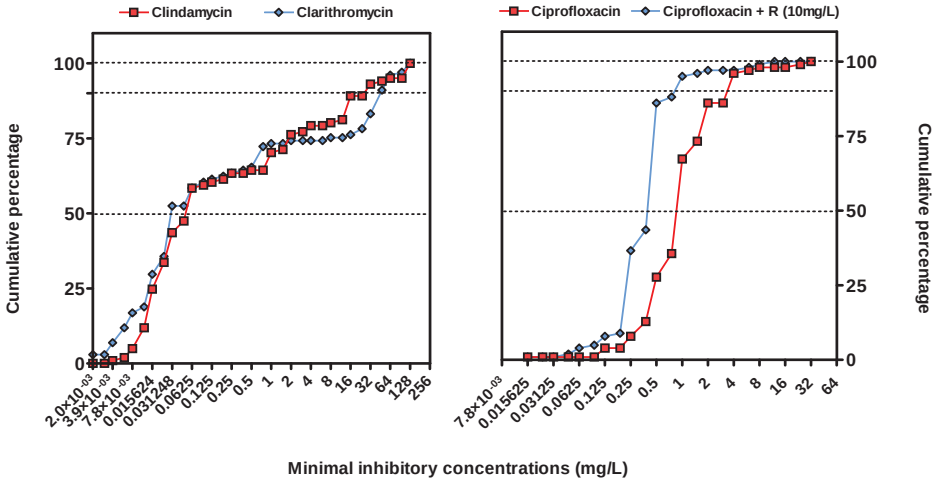
Supplementary Fig. S2. Minimum inhibitory concentration (MIC) distributions (cumulative percentages) of the macrolide and fluoroquinolone markers of efflux, respectively, for 101 non-duplicate *Streptococcus pneumoniae* isolates from chronic obstructive pulmonary disease patients: clindamycin versus clarithromycin (left panel) and ciprofloxacin versus ciprofloxacin + reserpine (R) (right panel). Three horizontal dotted lines are drawn at values corresponding to the MIC_{50}, MIC_{90} and MIC_{100} (MICs required to inhibit 50%, 90% and 100% of the isolates, respectively).

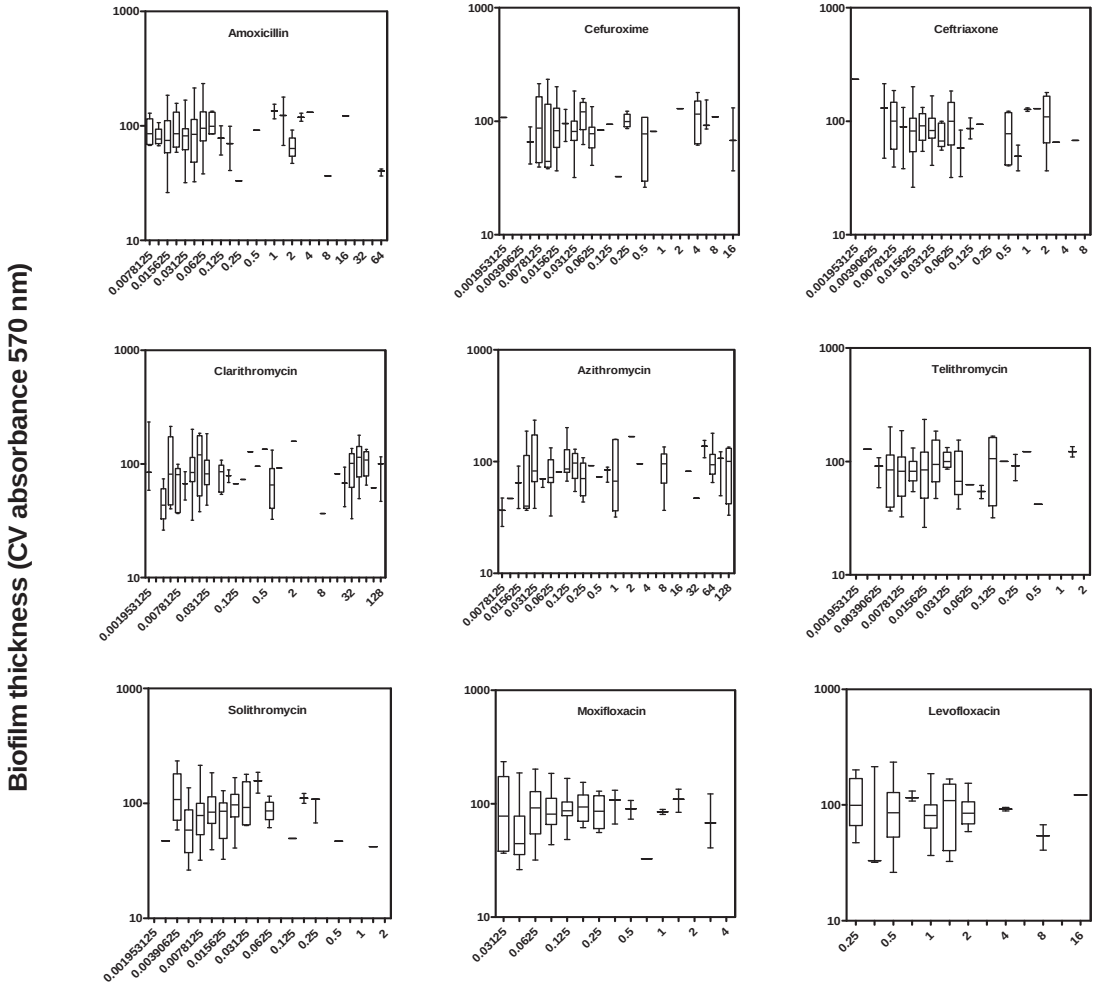
Supplementary Fig. S3. Box and whisker plots representing biofilm production after 10 days of culture for all isolates as a function of their minimum inhibitory concentration (MIC) in broth for three β-lactams (amoxicillin, cefuroxime and ceftriaxone), two macrolides (clarithromycin and azithromycin), two ketolides (telithromycin and solithromycin) and two fluoroquinolones (moxifloxacin and levofloxacin). Data are presented as box and whiskers plots giving the 25, 50 and 75 quartiles (boxes and horizontal line) and extending from 0 to 100% of the isolates. No significant correlation was seen between MIC and biofilm thickness [one-way analysis of variance (ANOVA) with or without Tukey's post-test]. CV, crystal violet.

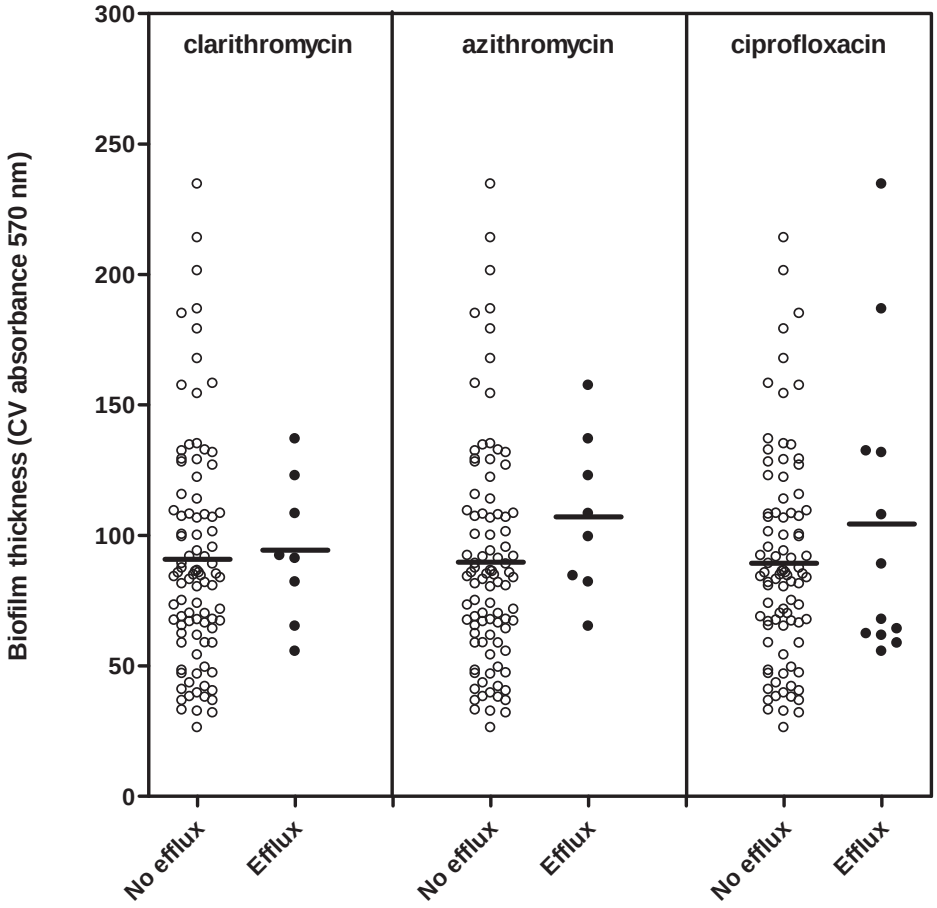
Supplementary Fig. S4. Distribution of isolate biofilm production as a function of phenotypic efflux. Comparison of biofilm production after 10 days of culture for strains resistant to both clarithromycin and azithromycin and to ciprofloxacin using

European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretive criteria (*Breakpoint tables for interpretation of MICs and zone diameters. Version 4.0*; http://www.eucast.org [accessed 2014]) and grouped according to the absence (open symbols) or presence (closed symbols) of efflux as detected for clarithromycin and azithromycin by dissociation of susceptibilities with clindamycin, and for ciprofloxacin by a two-fold decrease in minimum inhibitory concentrations upon addition of reserpine (10 mg/L). No correlation between efflux and biofilm thickness was seen (unpaired *t*-test, with or without Welch correction). CV, crystal violet.


Antwerp
East Flanders
West Flanders
Limburg
Flemish
Brabant
Walloon Brabant
Hainaut
Liège
Namur
Luxembourg
Serogroups with > 7.5 % incidence
in whole population (n=101)
• : Serogroup 6 (10.89 %)
• : Serogroup 23 (10.89 %)
• : Serogroup 11 (8.91 %)
• : Serogroup 19 (8.91 %)
• : Serogroup 15 (7.92 %)


Clindamycin
Clarithromycin
Ciprofloxacin
Ciprofloxacin + R (10mg/L)
Cumulative percentage
Cumulative percentage
Minimal inhibitory concentrations (mg/L)


Amoxicillin
Cefuroxime
Ceftriaxone
Clarithromycin
Azithromycin
Telithromycin
Solithromycin
Moxifloxacin
Levofloxacin
Biofilm thickness (CV absorbance 570 nm)
Minimal inhibitory concentrations (mg/L)


clarithromycin
azithromycin
ciprofloxacin
Biofilm thickness (CV absorbance 570 nm)
300
250
200
150
100
50
0
No efflux
Efflux
No efflux
Efflux
No efflux
Efflux

2. Models and methods

Such as mentioned in the description of the aims of this thesis and in the previous original paper, the pneumococcal ability to produce biofilm *in vitro* was investigated because of the high implication of this bacterial life mode in tissue colonization and infections recurrence, partly favored by the barrier and protective role played by the matrix in which bacterial cells are embedded, leading to decreased antibiotic activity and treatment failure observed *in vivo* and in clinical practice.

A main goal of this thesis project was therefore to study and compare the *in vitro* activity of different classes of antibiotics, already on the market or in phase 3 of clinical development, towards pneumococcal biofilm.

To achieve this, we first needed to set up two *in vitro* models mimicking a primary (naïve model) or a secondary infection (induced model) in terms of kinetic of matrix production. Secondly, some adaptation of existing methods or assays was required to evaluate the antibiotic activity on the biofilm thickness and bacterial viability within the matrix.

The *in vitro* models development and the adaptation of existing methods to pneumococcal biofilms are described in this second chapter and, for each case, are illustrated with results of, at least, one experimental and practical application.

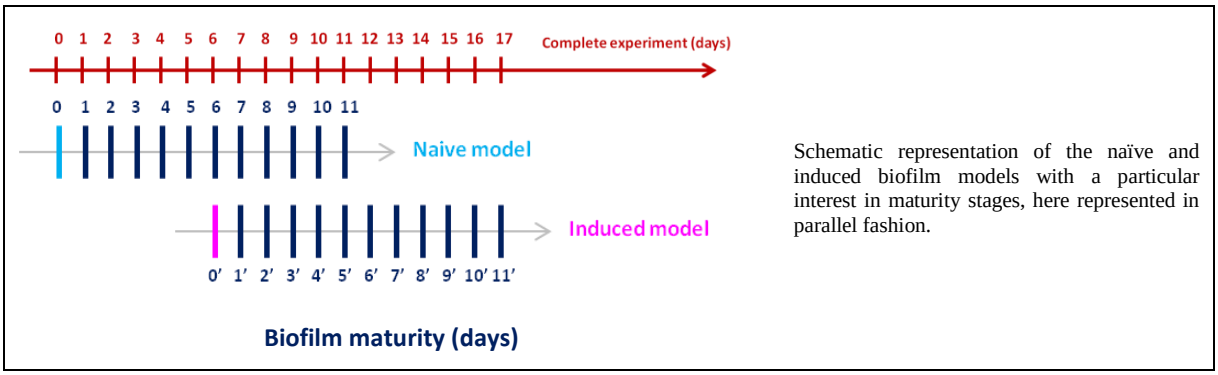
2.1. Naïve and induced static pneumococcal biofilm models

Naïve and induced biofilms were obtained through *S. pneumoniae* static culture in polystyrene 96-well plates using the growth medium recommended by the CLSI (caMHB supplemented with 5% of lysed horse blood, for Pneumococci MIC's measures, to which with also added 2% of glucose, because pilot experiments have shown that it allows a higher matrix production overtime (see Figure 27).

Naïve biofilms were made with the aim of having a model of pneumococcal biofilm primary attachment to a support. They were obtained using a bacterial culture in caMHB (OD_{620nm}: 0.1) composed of fresh bacterial colonies cultivated on blood agar plates containing 5% of defibrinated horse blood and added, in the culture plates, to the growth medium described here above (in proportion 1:7). The plates were then incubated during 2 to 11 days in a 5% carbon dioxide incubator, at 37°C and the culture medium was refreshed every two days.

Induced biofilms were obtained in the same way but, for this type of biofilms supposed to mimic more a secondary infection such as occurring during tissue colonization, the bacterial suspension consists in the supernatant of a 6-days old naïve biofilm taken from culture plates. Figure 27 offers a schematic representation of both biofilm models in parallel.

Figure 27



Schematic representation of the naïve and induced biofilm models with a particular interest in maturity stages, here represented in parallel fashion.

2.2. Method used for biofilm thickness quantification

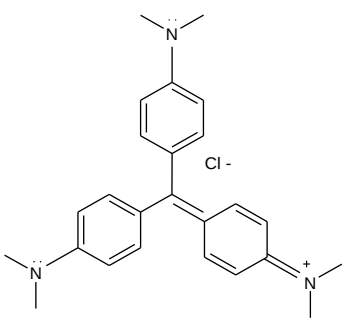
Biomass quantification over time was made through biofilm staining with crystal violet (CV), a cationic dye aspecifically binding to all negative charges present within the matrix (e.g. extracellular DNA) and on the surface of living and dead bacteria is commonly used in static models for biofilm thickness quantification.

In this work, we followed an already published protocol for pneumococcal *in vitro* biofilms consisting first in washing (with phosphate buffer saline [PBS]) and drying, at 60°C, biofilms stuck in the wells

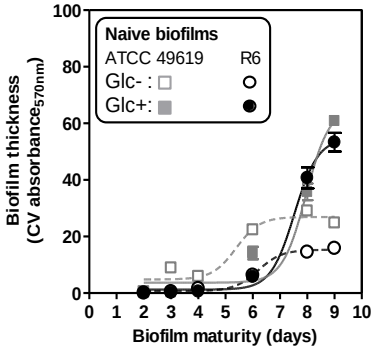
of the culture plates. Secondly the whole structure is stained by adding 175µl of crystal violet in each well for 10-minutes incubation at room temperature. After this step, the excess of non-bound dye is removed through washing under a tap water flow. Biofilms stained in violet are dried a second time at room temperature and finally, the staining is solubilized and homogenized by the addition of 200µl of 33% acetic acid per well to allow the biofilm thickness quantification through spectrophotometric measures of the crystal violet absorbance at 570nm. If the absorbance exceeds a value of 4 (the limit of detection of the microplates reader), dilutions of the plates content were performed with demineralized water.

Figure 28 shows the crystal violet structure (left panel), the biomass measured for naïve biofilms produced by two laboratory strains, ATCC49619 and R6, and developed in *S. pneumoniae* CLSI medium (caMHB supplemented with 5% of lysed horse blood) supplemented (Glc+) or not (Glc-) with 2% of glucose and finally, a comparison of biofilm thicknesses in naïve and induced models (right panel).

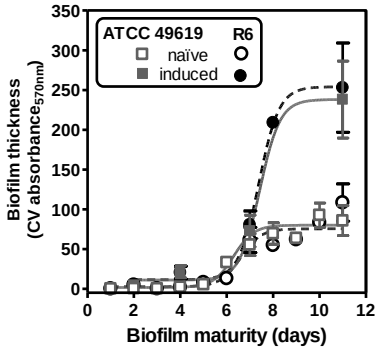
Figure 28



Crystal violet chemical structure



Evolution over time of matrix production (as evaluated by crystal violet [CV] absorbance) for naïve biofilms produced by the capsulated strain ATCC 49619 and the uncapsulated strain R6 by using growth media supplemented (Glc+, open symbols) or not (Glc-, closed symbols) with 2% of glucose. All values are means ± standard deviations (SD) of 4 independent determinations. When not visible, the SD bars are smaller than the size of the symbols.



Evolution over time of matrix production (as evaluated by crystal violet [CV] absorbance) by the capsulated strain ATCC 49619 (gray squares) and the non-capsulated strain R6 (black circles) in the naïve model (dotted lines, open symbols) and in the induced model (plain lines, closed symbols). The inset shows the same data at higher scale for the first 7 days of incubation. All values are means ± standard deviations (SD) of 8-28 independent determinations. When not visible, the SD bars are smaller than the size of the symbols.

During this thesis work, the influence of a wide variety of compounds on matrix production and biofilm adhesion were investigated using the crystal violet staining to better characterize pneumococcal *in vitro* biofilms. During these experiments, different interesting observations could be made. Next page offers a good illustration of biomass production overtime and matrix disassembly. To our knowledge, this phenomenon, well described for other bacteria such as *S. aureus* (Boles and Horswill, 2011), has never been reported for *S. pneumoniae* biofilms in the literature.

First demonstration of a matrix disassembly phenomenon in S. pneumoniae biofilms

This work was performed by Yvan Diaz Iglesias, a Master student I supervised. It has been presented during a reference meeting in Louvain-la-Neuve (Belgium) in December 2013 (illustrated in Figure 29).

Figure 29

Does nicotine influence the kinetics of biofilm development in reference and clinical strains *of Streptococcus pneumoniae* ?

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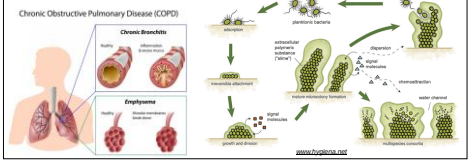
Introduction

- *S. pneumoniae* (SP) is a human pathogen frequently involved in acute exacerbations of chronic bronchitis (AECB).
- Biofilms are involved in persistent infections such as AECB.
- Tobacco smoking is one of the main risk factors to develop chronic bronchitis (**1**).

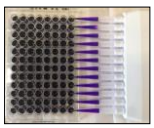


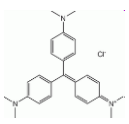
Aim of the study

- Our objective was to study the impact of nicotine on development of SP biofilm, using *in vitro* models of young to very mature biofilms.

Methods

- Strains : reference strain ATCC49619 and 2 clinical isolates, N1, coming from a smoking COPD patient and SP1337, isolated from a child suffering from CAP (non smoking patient).
- Biofilm : SP strains were cultivated for 2 to 16 days in 96-well plates using cation-ajusted Mueller Hinton broth with lysed horse blood (5%), glucose (2%) and supplemented or not with nicotine 15,3mg/L [human urinary concentration in smokers, **2**] as growth medium.
- Biofilm mass : measured by crystal violet staining and OD measurement at 570 nm (**3**).





References

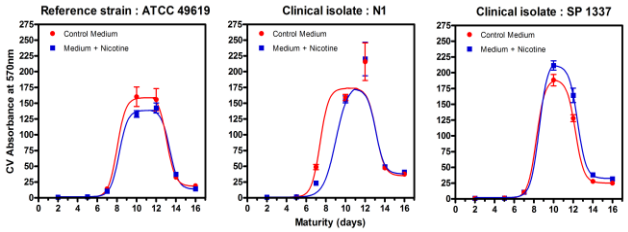
(1) Fabbri *et al.* (2008). *European Respiratory Journal*. 31, 204-212.

(2) Feyeraband *et al.* (1982). *British Medical Journal.* 284, 1002-1004.

(3) Roveta *et al.* (2007). *International Journal of Antimicrobial Agents*. 30, 415-421.

(4) Blaise *et al.* (2011). *Trends in Microbiology*. 19, 449-455.

Results



Kinetics of biofilm development in *S. pneumoniae* strains, as quantified by measurement of crystal violet OD_{570nm} (mean with SD, n=12)

- Biofilms developed overtime following a three-steps process.
  - Firstly, there is a lag phase of approx. 6-7 days during which there is no or minimal increase in biomass.
  - Secondly, biomass increased following a sigmoid function (with an Hillslope of 1) to reach a plateau at day 10, with the knee point of the curve being observed at day 7-8.
  - Thirdly, biomass decreased following again a sigmoid function (with a Hillslope of -1), to reach a lower plateau value at day 14.
- There was no significant difference in biomass between strains (one way-Anova with Tuckey post-test)
- The addition of nicotine did not significantly modify the extent or the rate of the whole process (unpaired t-test Welch corrected at each time point).

Conclusion

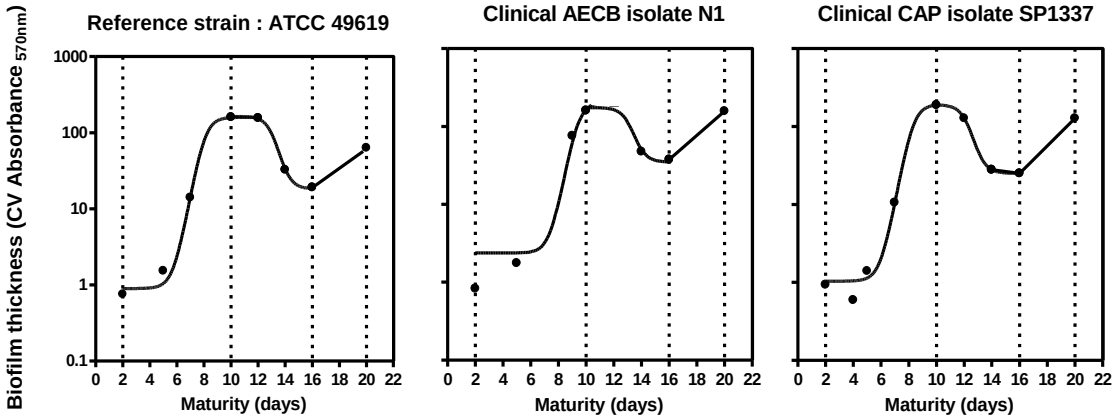
- Biofilm development in *S. pneumoniae* occurs after a latency phase, suggesting a slow adhesion of the bacteria to the support and/or a progressive adaptation of bacterial metabolism.
- A disassembling process seems to take place in very mature biofilms (> 12 days), which occurs at a rate similar to the assembling process.
  To our knowledge, this phenomenon, already described for other bacterial species (**4**), had previously never been observed in other *in vitro* models of pneumococcal biofilms. It may play a role in the spreading of the infection.
- Biofilm formation is not affected by the origin of the strain (smoker or non smoker), neither by the presence of nicotine in the culture medium. This may suggest that the preferential adhesion of bacteria in the respiratory tract of COPD patients may rely rather on the physiopathological environment to which bacteria are exposed *in vivo* than on a direct stress induced by nicotine on bacteria.

This poster will be made available for download after the meeting at http://www.facm.ucl.ac.be/posters.htm

Two main facts were presented in this poster. First, nicotine did not influence on pneumococcal biofilm matrix production overtime for a concentration relevant of those that can be found in smokers' airways and secondly, a disassembly phenomenon occurred from day 14 of maturity.

The matrix disassembly was later confirmed by myself through similar studies conducted until 20 days of maturity, using the same strains as biofilm producers such as illustrated in Figure 30.

Figure 30



Evolution over time of matrix production (as evaluated by crystal violet [CV] absorbance) for naïve biofilms produced by the capsulated reference strain ATCC49619, by the clinical isolate N1 collected in the sputum of an acute exacerbation of chronic bronchitis (AECB) patient and by the clinical isolate SP1337 coming from a 1-year old infant suffering from community acquired pneumonia (CAP). All values are means ± standard deviations (SD) of 2-8 independent determinations. When not visible, the SD bars are smaller than the size of the symbols.

On the basis of these biofilm thickness measures made overtime, it appears that the disassembly phenomenon, occurring between day 12 and 16 of maturity, is not a biofilm total loss or disintegration but rather a necessary step to allow the release of some bacterial step, responsible, *in vivo*, of the production of a second biofilm at some distance from the parent structure and therefore of tissue colonization (Vlastarakos *et al.*, 2007). Moreover, after this release of bacteria to the planktonic compartment, we clearly observe a regrowth in thickness from day 16, confirming the cyclic trend of matrix production already described in the literature.

2.3. Methods used to quantify the bacterial metabolism and viability

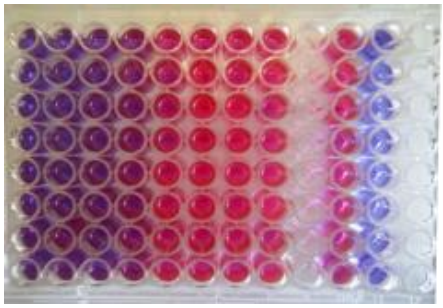
2.3.1. The Colony Forming Units (CFU) counting

The number of bacterial cells per volume in planktonic suspensions or biofilm supernatant was counted through plating of 100µl on blood agar plates, after serial dilutions (from 10^1 to 10^6 times) with water. Cell numbers were determined by colonies counting after 24 hours of culture at 37°C in a 5% carbon dioxide incubator.

2.3.2. The resazurin reduction assay

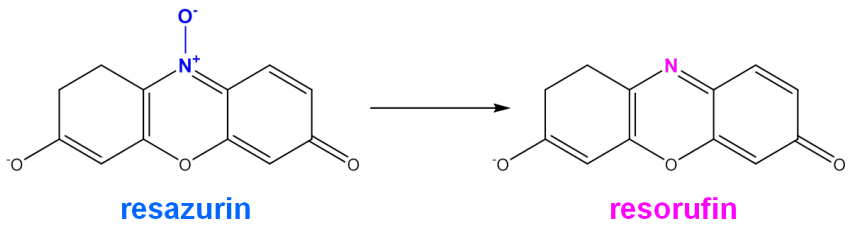
The bacterial viability and metabolic function could also be determined and followed overtime through measuring the reduction rate of resarurin, a blue and non-fluorescent dye, into resorufin, a pink and fluorescent compound (see Figure 31).

Figure 31



Picture of a 96-well plate containing a 4-days old naïve biofilm produced by the reference strain ATCC49619 and treated during 24h with a gradient of clarithromcycin.

After antibiotic treatment, sessile bacteria were incubated with resazurin 0.001% m/v during 32 hours to allow dye reduction into pink and fluorescent resorufin if viable cells are present within the matrix. The two right wells columns contain the negative control (C- ; growth medium without antibiotic) and positive control (C+, 1% sodium dodecyl sulfate solution) and the height left columns of wells contains the gradient of clarithromycin concentrations (diluted from 10 to 10, from 10^3 to 10^{-4} times the MIC in broth).



Resazurin and resorufin chemical structures with the reduction site marked in color.

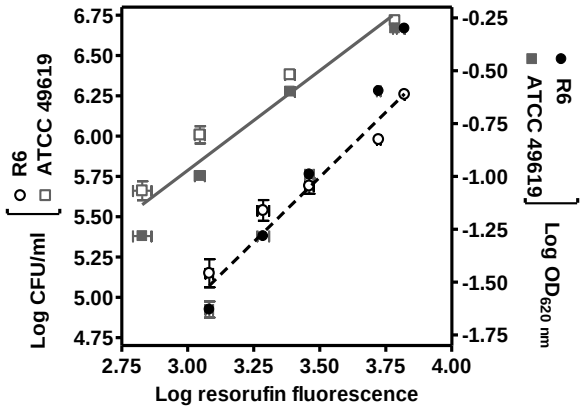
This assay has initially been adapted to *S. aureus* and *P. aeruginosa* biofilms by Tote and colleagues (Tote *et al.*, 2008a ; Tote *et al.*, 2010). The principle of the method is the following: after having removed the culture medium used for biofilm growth and washed the plates with PBS, sessile bacterial cells living within the matrix are incubated with resazurin, in the dark. Resazurin is then reduced into fluorescent resorufin overtime by metabolically active bacteria. We adapted it to our *in vitro* models of pneumococcal naïve and induced and to planktonic cultures. This required some modifications of initial experimental conditions and validation steps. They are described and illustrated hereafter.

After having confirmed the resorufin fluorescence spectrum (λ_{exc} 560nm ; λ_{em} 590nm), avoided possible fluorimetric measurements bias related to solvents and growth medium composition, pilot experiments were performed to determine the best resazurin concentration to engage. These experiments have allowed us to establish that a concentration equal to 0.001% m/v was optimal for our studies with *S. pneumoniae*. This solution was prepared by first making a 10-times concentrated stock solution by dissolving the resazurin sodium salt in PBS. This solution was then brought to the final concentration (0.001% m/v with caMHB) which was added in culture plates.

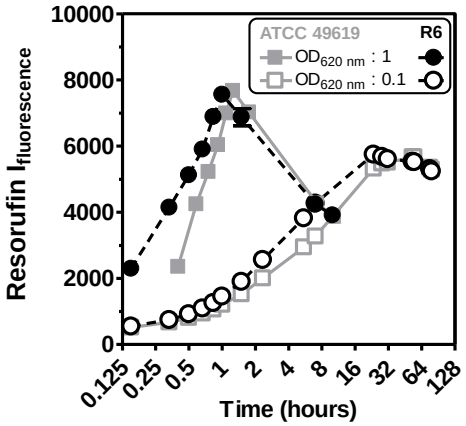
The main advantage of this enzymatic method is to rapidly obtain an accurate reflect of the number of viable and metabolically active cells, usually determined in planktonic cultures by CFU counting. Therefore, we validated our assay by (i) establishing correlations between the number of bacteria (CFU per volume unit), the optical density of the bacterial suspension at 620nm and the intensity of resorufin fluorescence and (ii) by measuring the signal of resorufin fluorescence overtime for planktonic cultures containing different bacterial loads and for sessile cells of young to very mature biofilms.

Figure 32 shows correlations between CFU counting, optical densities$_{620nm}$ and resorufin fluorescence signals measured for planktonic cultures made with two reference strains, ATCC 49619 and R6 (left panel) and the evolution of the resorufin fluorescence signal overtime for planktonic cultures made with the two same strains using different starting inocula (right panel).

Figure 32



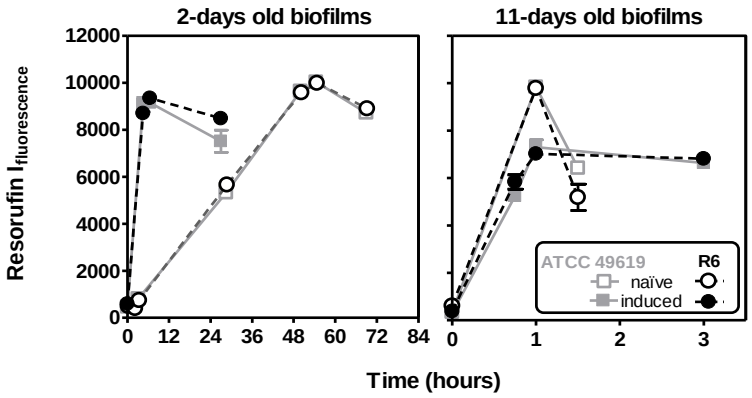
Correlation between resorufin fluorescence signal (after 3 h of incubation of planktonic cultures with 10 µg/ml resazurin) and bacterial inoculum as evaluated by the number of CFU (left axis) or the optical density of the suspension (right axis). Gray symbols and lines: ATCC49619; black symbols and lines: R6. Data are means ± SD of three independent determinations; when non visible, errors bars are smaller than the symbols.



Evolution of resorufin fluorescence overtime with the capsulated strain ATCC 49619 (gray squares) and the non-capsulated strain R6 (black circles) in planktonic cultures using starting inocula at OD_{620nm} = 0.1 (open symbols) or at $OD_{620\ nm}$ = 1 (plain symbols). All values are means ± standard deviations (SD) of 3 independent determinations. When not visible, the SD bars are smaller than the size of the symbols.

Because the maximal resorufin fluorescent signal has been shown to be directly dependent on the number of viable bacteria, it is easy to understand that the time necessary to obtain the maximal fluorescence value varies with the initial inocula in planktonic cultures and with biofilm maturity for sessile cells. Figure 33 shows the evolution overtime of the resorufin fluorescence signal measured for young and mature naïve and induced *in vitro* pneumococcal biofilms.

Figure 33



Evolution of resorufin (RF) fluorescence overtime with the capsulated strain ATCC 49619 (gray squares) and the non-capsulated strain R6 (black circles) 2 and 11 day-old naïve (open symbols) and induced (closed symbols) biofilms, respectively middle and right panels. All values are means ± standard deviations (SD) of 3 independent determinations. When not visible, the SD bars are smaller than the size of the symbols.

Over the incubation time, metabolically active and viable pneumococcal sessile cells reduce the non-fluorescent resazurin into fluorescent resorufin. This phenomenon is therefore characterized by an increase of the resorufin intensity of fluorescence over time until reaching a maximal value. Such as illustrated in Figure 31, this maximum of fluorescence is closely linked to the number of viable cells and therefore, may be used to reflect the rate of antibiotic killing activity.

The knowledge of the incubation times required to reach this maximal fluorescence is essential to perform pharmacodynamics studies of antibiotic activity and to compare different conditions (*e.g.* the rate of cell killing induced by different antibiotic concentrations) with the highest possible fluorimetric sensitivity.

For each biofilm model, each maturity stage and each strain, intensities of resorufin fluorescence were therefore recorded after the same periods (reaching from 10 minutes to 72 hours) of biofilm incubation in the presence of resazurin. These measures were reproduced during 8 individual preliminary pilot experiments. For each condition, we assessed that fluorescence intensities after the same incubation time were not statistically different (One-way ANOVA with Tukey post-test). Through these kinetic studies, we could precisely determine, for each condition, which were the biofilms incubation times in the presence of resazurin (Mean ± SD) required to reach the maximal resorufin fluorescence signals.

The intra-experiment mean values of incubation times of 2-, 4-, 7, and 11-days old ATCC49619 and R6 biofilms are mentioned in Table 5.

Table 5. Incubation times needed to reach maximal resorufin fluorescence signals for naïve and induced ATCC 49619 and R6 biofilms of different maturity stages.

Biofilm maturity (days)	Incubation time with resazurin before fluorescence reading (hours)[a]	
	naïve model	induced model
2	56	4.5
4	32	2
7	2	1
11	1	1

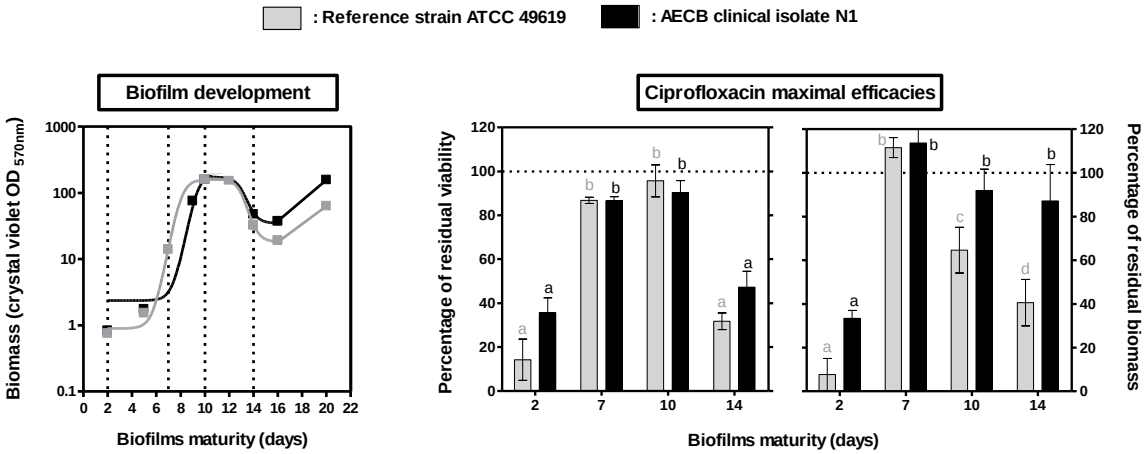
The resazurin viability assay was used for different pharmacodynamic studies such as for those investigating the impact of spontaneous biofilm disassembly, illustrated in Figure 29, on the antibiotic activity towards sessile bacteria.

Impact of spontaneous biofilm disassembly on the antibiotic activity

The impact of spontaneous biofilm disassembly on antibiotic activity was investigated using very mature naïve biofilms. Indeed, such as previously mentioned and illustrated in Figure 30, biofilm production is probably a cyclic process, mainly composed of bacterial attachment to a support and intensive matrix production, followed at very old maturity stages by spontaneous matrix disassembly. Because matrix thickness is a determinant modulatory factor for antibiotic activity, we investigated if spontaneous disassembly occurring after 14 days of culture could restore the biofilm susceptibility to antimicrobials. This study, such as kinetics studies of disassembly, was also initiated in the context of the work of a Master student in toxicology I supervised, Yvan Diaz Iglesias, and results will be presented during the next meeting of the ESCMID Study Group for Biofilms (ESGB) that takes place in Rome in October 2014.

Data showed that, even for poorly active molecules such as ciprofloxacin, matrix disassembly enables to highly improve antibiotic killing activity on sessile cells. Results obtained with this fluoroquinolone towards biofilms made reference strain ATCC49619 and one of the new clinical isolates, strain N1, collected in the sputum of a GOLD-3 COPD patient hospitalized for infectious exacerbation are illustrated on next page in Figure 34.

Figure 34



Detection of a matrix disassembly phenomenon improving the antibiotic killing activity towards very mature *S.pneumoniae* (Sp) *in vitro* biofilms.Y. Diaz Iglesias, F. Van Bambeke and N. M. Vandevelde (ESGB, 2014).

Left panel: Evolution of biofilm production (as evaluated by crystal violet [CV] absorbance) overtime in a naïve model and by the capsulated reference strain ATCC49619 (in gray) and AECB clinical isolate N1 (in black). All values are means ± standard deviations (SD) of 2-8 independent determinations. When not visible, the SD bars are smaller than the size of the symbols. Middle and right panels: Maximal ciprofloxacin efficacies on pneumococcal survival (resorufin fluorescence; middle) and biomass thickness (crystal violet absorbance; right panel) as compared to controls (no antibiotic added) for 2-, 7-, 10 and 14-days old naive biofilms of strain ATCC49619 and N1. Results are expressed in percentage of residual viability and biomass after 24h of incubation in the presence of a ciprofloxacin gradient with values calculated as means **±** SEM 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. Statistical analyses: one-way ANOVA with Tukey post-test for multiple comparisons between different maturity stages; values with different letters are significantly different from each other ($p<0.05$).

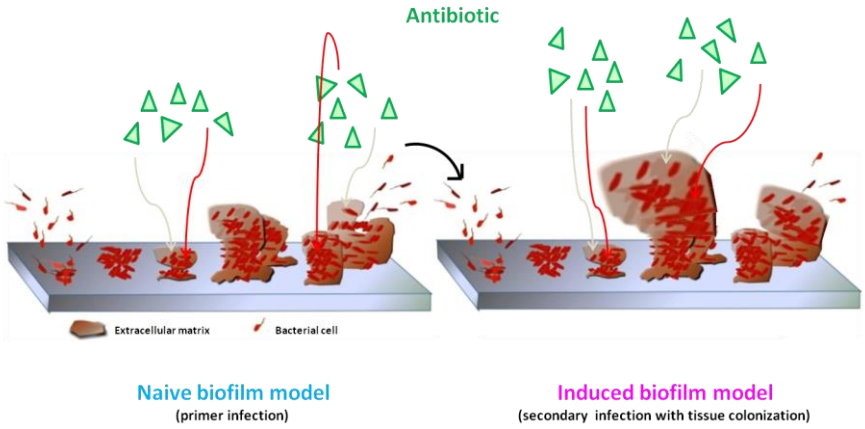
For both strains, biomass firstly increased from day 2 (CV_{OD} = 0.8 ± 0.04) until reaching a plateau of maximal thickness at day 10 to 12 (CV_{OD} = 172.8 ± 24.9). From day 12 to 14, matrix loss occurred with a second plateau phase (characterized by less biomass) obtained between days 14 to 16 (CV_{OD} = 34.2 ± 10.2). From day 16 to 20, biofilm growth resumed until CV_{OD} = 110 ± 46.7 (day 20). Maximal percentages of ciprofloxacin activity (E_{max}) towards 2-days old biofilms were equal to 86-65 (towards bacterial survival and biomass) and decreases until day 10 with only 14% and 0% of activity on survival and biomass respectively. However, at day 14, the matrix loss observed under control conditions (no antibiotic) was accompanied by killing activity levels recovering the same values as towards young (2-days) biofilms. Concerning the ability of ciprofloxacin to remove biomass from the plates, an improvement of efficacy was only observed for biofilms made by strain ATCC49619, with 60% of maximal efficacy. For biofilms made by AECB clinical strain N1, a similar trend was observed but without significant improve, compared to day 10. This may perhaps be related to the slightly higher biomass amount measured for 14-days old biofilms produced by this isolate.

In conclusion, we can say that biomass amount strongly affects ciprofloxacin killing activity against *Streptococcus pneumoniae* biofilms but that spontaneous matrix loss, occurring from day 14 and probably related to disassembly, allows a significant restoration of activity on old biofilms and that agents promoting this phenomenon are promising to deal with infections recurrence.

3. *In vitro* pharmacodynamic studies of antibiotic activity in models of pneumococcal biofilms

Through the epidemiological study conducted on a Belgian COPD population suffering from acute exacerbations episodes caused by pneumococcal infections of the airways, we could observe that all isolates were able to produce biofilms, some isolates producing more matrix than others.

Because of the well-known implication of this bacterial life mode in the chronicity of infections, partly explained by the protective role played by the matrix against the antibiotic pressure, we decided to investigate the activity of different antibiotic classes on biomass and bacterial survival in young to very mature naïve and induced biofilm models, characterized in the previous chapter by possessing different thicknesses. Methods used for the quantification of the antibiotic activity are the biofilm staining with crystal violet and the resazurin viability assay and have also been described previously.



AUTHORS' CONTRIBUTIONS:

1. Conceived and designed experiments: NMV, FVB;
2. Performed experiments: NMV;
3. Analyzed data: NMV, PMT, FVB;
4. Wrote the paper: NMV, PMT, FVB.

Antimicrobial Agents and Chemotherapy

Antibiotic Activity against Naive and Induced Streptococcus pneumoniae Biofilms in an *In Vitro* Pharmacodynamic Model

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Antibiotic Activity against Naive and Induced *Streptococcus pneumoniae* Biofilms in an *In Vitro* Pharmacodynamic Model

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Biofilms play a role in the pathogenicity of pneumococcal infections. A pharmacodynamic *in vitro* model of biofilm was developed that allows characterization of the activity of antibiotics against viability and biomass by using in parallel capsulated (ATCC 49619) and noncapsulated (R6) reference strains. Naive biofilms were obtained by incubating fresh planktonic cultures for 2 to 11 days in 96-well polystyrene plates. Induced biofilms were obtained using planktonic bacteria collected from the supernatant of 6-day-old naive biofilms. Biomass production was more rapid and intense in the induced model, but the levels were similar for both strains. Full concentration responses fitting sigmoidal regressions allowed calculation of maximal efficacies and relative potencies of drugs. All antibiotics tested (amoxicillin, clarithromycin, solithromycin, levofloxacin, and moxifloxacin) were more effective against young naive biofilms than against old or induced biofilms, except macrolides/ketolides, which were as effective at reducing viability in 2-day-old naive biofilms and in 11-day-old induced biofilms of R6. Macrolides/ketolides, however, were less potent than fluoroquinolones against R6 (approximately 5- to 20-fold-higher concentrations needed to reduction viability of 20%). However, at concentrations obtainable in epithelial lining fluid, the viabilities of mature or induced biofilms were reduced 15 to 45% (amoxicillin), 17 to 44% (macrolides/ketolides), and 12 to 64% (fluoroquinolones), and biomasses were reduced 5 to 45% (amoxicillin), 5 to 60% (macrolides/ketolides), and 10 to 76% (fluoroquinolones), with solithromycin and moxifloxacin being the most effective and the most potent agents (due to lower MICs) in their respective classes. This study allowed the ranking of antibiotics with respect to their potential effectiveness in biofilm-related infections, underlining the need to search for still more effective options.

Biofilm has been defined as a "microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substance that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (1). Biofilms are now considered to play a major role in pathogenesis, with more than 60% of all human bacterial infections possibly being associated with microbial growth within this type of structure (2). Persistence or recurrence of biofilm-associated infections may stem not only from their role as a reservoir for secondary bacterial dissemination (2, 3) or their interference with the host's responses (prevention of phagocytosis) (4, 5) but also from their capacity to impair antibiotic action. Possible factors decreasing antibiotic activity include diffusion barrier effects and phenotypic or metabolic variations accompanying the switch from a planktonic to a sessile mode of life that reduce their susceptibility to antibiotics (6–8).

Biofilms can develop on artificial surfaces, like medical devices, but also on tissues or mucus, as observed, for example, with *Streptococcus pneumoniae* in nasopharynx colonization (9), otitis media (9–11), or chronic rhinosinusitis (12). Therefore, *in vitro* (13–18) and *in vivo* (11, 19, 20) models of pneumococcal biofilms have been developed and used to study the pathophysiology of the infection as well as the activities of the antibiotics. None of these studies, however, developed a comprehensive and comparative pharmacodynamic model of the activity of antibiotics against biofilms of *S. pneumoniae*. Moreover, they focused on short maturity stages (14, 17, 21–25) that are probably poorly representative of the types of biofilms that develop in chronic infections or in infections occurring in deep airways (25, 26).

In the present work, we have set up *in vitro* models of pneumococcal biofilms at both young and old maturity stages in an attempt to mimic what takes place during short- and long-term infections by *S. pneumoniae*. The first model consists of naive biofilms, in which freshly grown bacteria are allowed to adhere on multiwell plates and to form a biofilm for up to 11 days. A second model consists of induced biofilms, in which bacteria collected from the supernatant of naive biofilms are used as a starting inoculum. This model may better take into account the adaptative process mediated by the quorum sensing molecules that takes place during biofilm maturation (27, 28) and which was already well demonstrated to take place with clinical isolates form other bacterial species (29, 30). The models have been tested with antibiotics representative of the 3 main classes of antibiotics active against *S. pneumoniae*, namely, amoxicillin (for β-lactams), clarithromycin (for macrolides), and levofloxacin and moxifloxacin (for fluoroquinolones). We also included solithromycin, a fluoroketolide active against macrolide-resistant strains (31) that has successfully completed phase II clinical trials in moderate to moderately severe community-acquired pneumonia (32).

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* (Klein) Chester reference strain ATCC 49619 (capsulated [serotype 19 F]; isolated from the sputum of a 75-year-old male) (33) and R6 ATCC BAA-255 (uncapsulated; derived from the capsulated clinical isolate D39) (34–36) were grown on Mueller-Hinton blood agar plates supplemented with 5% defibrinated horse blood at 37°C in a 5% CO_2 atmosphere.

Antibiotics. The tested antibiotics were obtained as microbiological standards from the following sources: clarithromycin (potency, 100%) from Teva Laboratories (Paris, France), solithromycin (potency, 100%) from Cempra Pharmaceuticals (Chapel Hill, NC), levofloxacin hemihydrate (potency, 97.5%) from Sanofi-Aventis Deutschland GmbH (Frankfurt, Germany), and moxifloxacin chlorhydrate (potency, 90.9%) from Bayer Schering Pharma AG (Berlin, Germany). Amoxicillin (potency, 100%) was procured as the corresponding branded product for human parenteral use distributed for clinical use in Belgium as Clamoxyl iv/im by GlaxoSmithKline s.a/n.v (Genval, Belgium). Sterile stock solutions of each antibiotic were prepared according to the manufacturer's instructions.

Susceptibility testing. MICs were determined by microdilution following the guidelines of the Clinical and Laboratory Standards Institute (37) using cation-adjusted Mueller-Hinton broth (CA-MHB) (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 5% lysed horse blood, starting from overnight bacterial cultures diluted to an optical density at 620 nm (OD_{620}) of 0.08 to 0.1 (corresponding to 0.5 McFarland standard). MICs were read after 18 to 24 h of incubation at 37°C.

Development of naive and induced biofilm models. Ninety-six-well plates (European catalog no. 734-2327; VWR, Radnor, PA) were used as the support for the biofilm growth. In each well, 25 μl of bacterial culture (OD_{620} of 0.1) was added aseptically to 175 μl of cation-adjusted Mueller-Hinton broth (Becton, Dickinson Company, Franklin Lakes, NJ) supplemented with 5% lysed horse blood and 2% glucose (Sigma-Aldrich, St. Louis, MO). In preliminary experiments, we showed that biofilm formation was increased if CA-MHB supplemented with 5% lysed horse blood is used instead of Todd-Hewitt broth supplemented with 0.5% yeast extract and, for both of these media, by addition of 2% glucose. Under these conditions, the initial inoculum was approximately 5×10^7 CFU/ml ($4.92 \pm 1.22\ 10^7$ CFU/ml for strain ATCC 49619 and $5.18 \pm 0.64\ 10^7$ CFU/ml for strain R6 in preliminary experiments [in triplicate from 2 independent pilot experiments]). The naive model of biofilm was obtained by incubating these plates for 2 to 11 days with medium replacement every 48 h. The induced model was produced by starting with an inoculum of 25 μl of the supernatant (free bacteria) from a 6-day-old biofilm, corresponding to an initial bacterial density of approximately 8.5×10^7 to 9×10^7 CFU/ml ($8.5 \pm 0.4\ 10^7$ CFU/ml for strain ATCC 49619 and $8.9 \pm 1.1\ 10^7$ CFU/ml for strain R6, respectively, in preliminary experiments [in triplicate from 2 independent pilot experiments]). Biofilm culture was then performed as for the naive model. All cultures were incubated in a 5% CO_2 atmosphere.

Determination of biofilm mass (crystal violet staining). Biofilm mass was evaluated by measuring the absorbance of crystal violet, a cationic dye that quantitatively stains nonspecifically negatively charged biofilm constituents based on ionic interactions (38). After elimination of the medium, wells were washed once with phosphate-buffered saline (PBS) and dried for 1 h at 60°C, after which 150 μl of crystal violet (2.3% solution in 20% ethanol [Sigma-Aldrich, St. Louis, MO]) was added to each well and left at room temperature for 10 min. After the stain had been poured out, wells were washed under running water for 5 min, and the plates were dried. The dye bound to the plate was solubilized and homogenized by 1 h of incubation with 200 μl of 33% acetic acid. The absorbance of each well was measured at 570 nm using a microplate spectrophotometer (VersAmax Tunable microplate reader; Molecular Devices, Sunnyvale, CA).

Determination of bacterial viability within the biofilm by using resazurin. Viability was determined using resazurin, a blue phenoxazin dye that is reduced by viable bacteria to the pink fluorescent compound resorufin (39, 40). After elimination of the medium and washing of the wells with PBS at room temperature, 200 μl of a 0.001% resazurin (Sigma-Aldrich) solution in CA-MHB was added to each well. Plates were then incubated at room temperature in the dark, and fluorescence was measured ($\lambda_{excitation}$, 560 nm; $\lambda_{emission}$, 590 nm) thereafter using a microplate spectrofluorometer (SPECTRAmax Gemini XS; Molecular Devices). Preliminary experiments were done to determine the optimal time of incubation before plates were read (see Results and Table 1).

TABLE 1 Time of incubation with resazurin needed to obtain a maximal fluorescence signal for biofilms of increasing maturity

Biofilm maturity (days)	Incubation time before fluorescence reading (h) in[a]:	
	Naive model	Induced model
2	56	4.5
4	32	2
7	2	1
11	1	1

[a] Shown are the incubation times necessary to reach the resorufin (RF) maximal fluorescence values measured by fluorimetry during kinetic studies (such as those illustrated for maturity stages of 2 and 11 days in Fig. 2). The studies were done using microplates containing naive and induced biofilms of strains ATCC 49619 and R6, which had maturity stages of 2, 4, 7, and 11 days. For each strain, the values are means of 4 to 8 independent determinations.

Antibiotic activity on bacterial viability within the matrix and on biofilm mass. At specific stages of biofilm maturity, the culture medium (including unbound planktonic bacteria) was removed and replaced with fresh medium (control), medium supplemented with antibiotics at concentrations ranging from 10^{-4}- to 10^3-fold their MIC (in order to obtain full concentration-effect relationships and calculate with accuracy the relevant pharmacodynamic parameters), or 1% sodium dodecyl sulfate (SDS), used as a positive control (full destruction of the biofilm and bacterial death) (41). After 24 h of incubation, the biofilm mass and the bacterial viability were measured using the crystal violet and resazurin assays, with data expressed as a percentage of the control value, using the formula $[(value_{AB} - value_{SDS})/(value_{CT} - value_{SDS})] \times 100$, where $value_{AB}$, $value_{SDS}$, and $value_{CT}$ are the absorbance or fluorescence signals recorded for biofilms incubated with antibiotic, SDS, or control medium, respectively.

Curve fitting and statistical analyses. Curve fitting analyses were made using Graph-Pad Prism version 4.03 (GraphPad Software, San Diego, CA). Data were used to fit a sigmoid function (Hill equation, slope factor set to 1) by nonlinear regression. The fitted function was then used to determine two key pharmacodynamic descriptors of antibiotic activity, namely, (i) the relative maximal efficacy (E_{max}; maximal reduction in biofilm mass or viability as extrapolated for an infinitely large antibiotic concentration) and (ii) the relative potency (C_{20} or C_{50}, i.e., the antibiotic concentration needed to achieve 20 or 50% reduction in bacterial viability within the biofilm or in biofilm mass). Confidence intervals at 95% (95% CI) and the standard errors of the mean (SEM) for the parameters of the Hill equation (E_{min}, E_{max}, and 50% effective concentration [EC_{50}]) were obtained from GraphPad. SEM on log C_{20} were calculated as $[\log C_{20\,(+5\%)} - \log C_{20\,(-5\%)}]/(2 \times 1.96)$, where $C_{20\,(+5\%)}$ and $C_{20\,(-5\%)}$ are the concentrations yielding a 20% reduction in signal as calculated from the equations of the curves delimiting the 95% CI. Statistical analyses were performed with Graph Pad Instat version 3.06 (GraphPad Software).

RESULTS

Characterization of biofilm formation in the naive and induced models and validation of the methods of assay (biomass and viability). In the first series of experiments, we compared the increases in biofilm mass over time for strains ATCC 49619 (capsu-

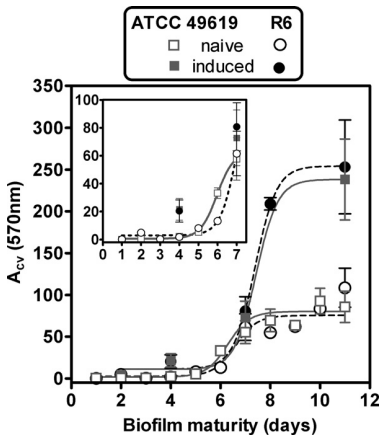

FIG 1 Evolution over time of matrix production (as evaluated by crystal violet [CV] absorbance) by the capsulated strain ATCC 49619 (gray squares) and the noncapsulated strain R6 (black circles) in the naive model (dotted lines, open symbols) and the induced model (solid lines, closed symbols). The inset shows the same data at higher scale for the first 7 days of incubation. All values are means ± standard deviations (SD) of 8 to 28 independent determinations. When not visible, the SD bars are smaller than the size of the symbols.

TABLE 2 MICs of antibiotics against the strains used in this study

Antibiotic by class	MIC (mg/liter) for: ATCC 49619	R6
β-Lactams		
Amoxicillin	0.064	0.032
Macrolides/ketolides		
Clarithromycin	0.032	0.064
Solithromycin	0.008	0.004
Fluoroquinolones		
Levofloxacin	1	0.5
Moxifloxacin	0.125	0.064

lated) and R6 (uncapsulated). Figure 1 shows that with fresh planktonic cultures (naive model), crystal violet staining started to exponentially increase after about 5 days to reach an apparent plateau at day 8. This suggests that bacteria at days 6 to 8 are probably in a metabolic state that actively produces biofilm. We therefore developed a second model (referred to as the "induced" model), in which biofilm growth was initiated using planktonic bacteria collected from the supernatant of 6-day-old biofilms. With these bacteria, the biofilm mass started to increase after only 4 days to reach, after 8 to 9 days, a value that was 3 times higher than that of the naive model.

In the second series of experiments, we validated our viability assay based on the reduction of resazurin into resorufin. The rate at which this reduction occurs is indeed dependent on both the biofilm maturity and the number of metabolically active bacteria, but the reaction product may undergo additional enzymatic and nonenzymatic transformation(s) (42, 43), causing the fluorescent signal to increase and then decrease over time. Figure 2 shows the change in fluorescence recorded over time upon incubation at room temperature for (i) planktonic cells at different densities, and (ii) sessile cells in (a) young (2 days) and old (11 days) biofilms and (b) naive and induced biofilms. Under all conditions, the signal increased until it reached a maximal value, after which fluorescence remained stable (low-density planktonic culture or old induced biofilms) or decreased. The time needed to reach the maximal value was much longer for (i) planktonic cells at low density versus high density (left panel), (ii) young biofilms versus old biofilms (compare middle and right panels), and (iii) young naive versus young induced biofilms (middle panel). Accordingly, and for all subsequent experiments, fluorescence recordings were made at the fixed times shown in Table 1, based upon the type of sample examined. Using crystal violet staining, we checked that no biofilm growth occurred during incubation with resazurin, even when prolonged for more than 48 h, probably due to the fact that incubation with resazurin was performed in CA-MHB, which is not appropriate for growth as a biofilm.

Susceptibility testing. The MICs of the antibiotics under study for the strains ATCC 49619 and R6 are shown in Table 2. Both strains were highly susceptible to all antibiotics, with solithromycin and levofloxacin demonstrating the highest and lowest activities, respectively.

Activities of antibiotics against biofilms (viability and biofilm mass). In the viability and biofilm mass experiments, we measured the effect of antibiotics on bacterial survival and biofilm

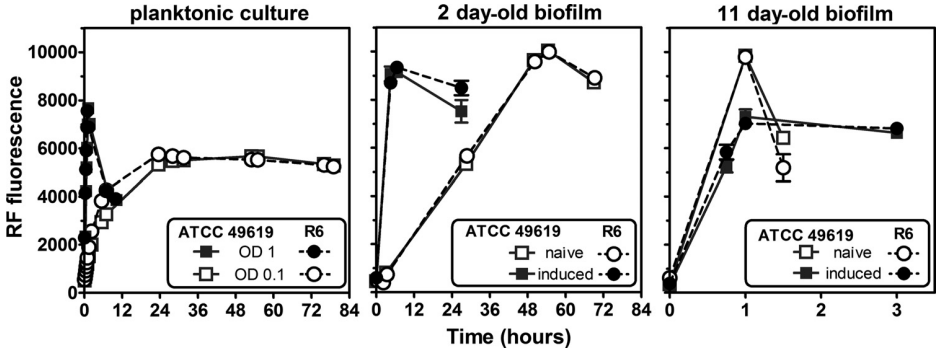

FIG 2 Evolution of resorufin (RF) fluorescence overtime with the capsulated strain ATCC 49619 (gray squares) and the noncapsulated strain R6 (black circles) in planktonic cultures (left panel) using starting inocula at an OD_{620} of 0.1 (open symbols) or 1 (closed symbols) or in biofilms (middle and right panels) at different maturity stages (2- and 11-day-old naive [open symbols] and induced [closed symbols] models). All values are means ± standard deviations (SD) of 3 independent determinations. When not visible, the SD bars are smaller than the size of the symbols.

mass after 24 h of incubation. We systematically compared naive and induced biofilms and, in each of these two models, the effects seen with young (2 days old) and old (11 days old) biofilms. Antibiotics were added to the medium over a wide range of concentrations to obtain full concentration-effect responses. Sigmoidal functions (Hill equations) with a slope factor of 1 could be fitted to all sets of data when plotted against the $\log_{10}$ value of the antibiotic concentration, which allowed direct comparison of the antibiotic maximal efficacies (E_{max}) and their relative potencies (C_{20} or C_{50}). Graphical representations are shown in Fig. 3 (amoxicillin), 4 (solithromycin), and 5 (moxifloxacin), with additional antibiotics presented in the supplemental material (clarithromycin in Fig. S1 and levofloxacin in Fig. S2). Pharmacodynamic parameters evaluating relative efficacy and relative potency are compared in a pictorial fashion in Fig. 6 and 7, with numerical data provided as supplemental material (see Tables S1 and S2 for the ATCC 49619 and R6 strains, respectively).

Considering first the effect of antibiotics on viability (left panels) and focusing on efficacy (maximal effect), we see that all antibiotics were globally most effective against 2-day-old naive biofilms, with a loss of viability ranging from 35% (clarithromycin for R6) to 81% (moxifloxacin) compared to control values (no antibiotic added). Eleven-day-old naive biofilms and 2- or 11-day-old induced biofilms showed much less reduction of viability that did not exceed approximately 40% for amoxicillin, clarithromycin, and solithromycin and reached 32 to 65% for levofloxacin and moxifloxacin, respectively. No systematic difference in efficacies was observed between biofilms formed with the ATCC 49619 and those with R6, except again for macrolides (clarithromycin and solithromycin), which were poorly active against biofilms formed with the R6 strain, even if young and naive. Examination then of relative potencies, showed that fluoroquinolones were more potent (lower C_{20} values, close to the MIC) than the other drugs against 2-day-old naive biofilms, while macrolides/ketolides were systematically less potent against 2-day-old induced or 11-day-old naive biofilms of strain ATCC 49619. C_{50} values were much higher or could not be determined under most conditions.

Considering now the activity of antibiotics on biomass (right panels), we globally see that the effects, although developing often in parallel to those described for viability, resulted in much lower maximal efficacy (no more than 50% reduction) for amoxicillin and clarithromycin, whatever the condition. Solithromycin showed a much larger maximal efficacy than clarithromycin against ATCC 49619, except against 11-day-old induced biofilms. Conversely, no systematic difference was seen for strain R6 between these 3 antibiotics. Globally, fluoroquinolones were the most active at reducing the biomass of young biofilms (especially moxifloxacin), but this difference from the other antibiotics was not maintained with 11-day-old biofilms in either naive or induced models. Against 2-day-old biofilms, C_{20} values were globally similar to those observed against viability, with only amoxicillin and solithromycin showing slightly higher potencies (lower C_{20} values) against biomass. Against 11-day-old biofilms, potencies were globally low; in many cases, a 20% reduction was not reached even at the highest antibiotic concentration tested.

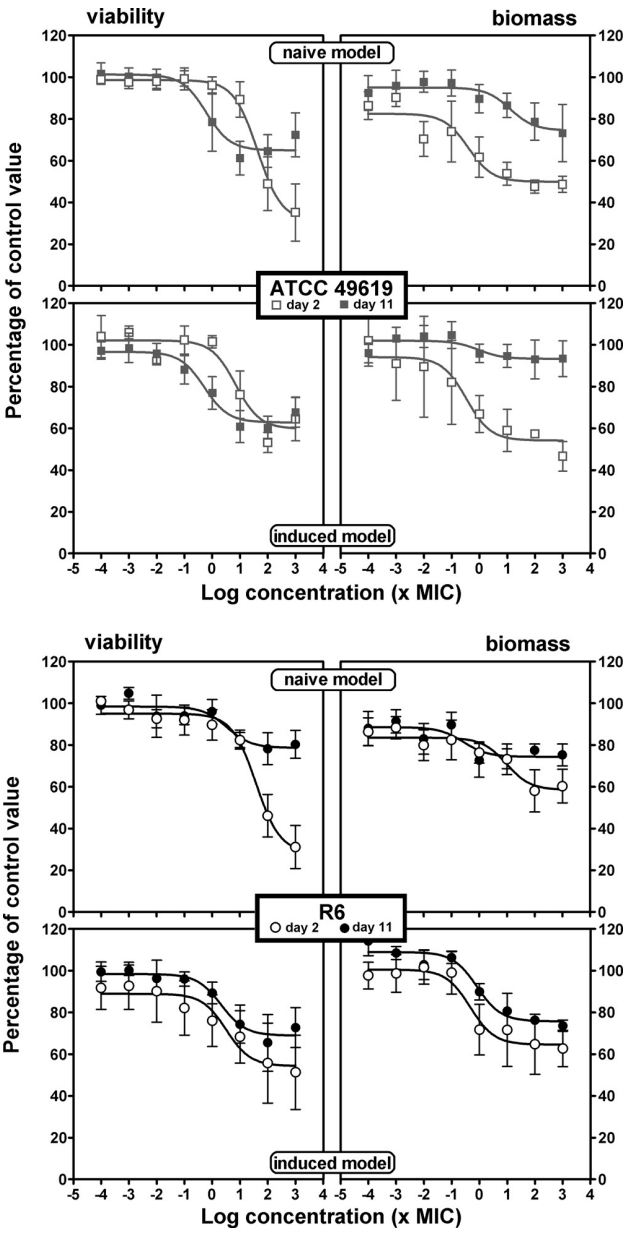

FIG 3 Concentration-response activity of amoxicillin against biofilms of ATCC 49619 (top) or R6 (bottom). Two-day-old (open symbols) or 11-day-old (closed symbols) biofilms from the naive model (upper panels for each strain) or the induced model (lower panels for each strain) were incubated with increasing concentrations of antibiotics for 24 h. The ordinate shows the change in viability (measured by the decrease in resorufin fluorescence [left panels]) or in biofilm mass (measured by the decrease in crystal violet absorbance [right panels]) as a percentage of the control value (no antibiotic present). All values are means ± SEM of 4 to 10 independent experiments performed in quadruplicate. When not visible, the error bars are smaller than the size of the symbols. The pertinent pharmacological descriptors of the curves are presented in Tables S1 and S2 in the supplemental material.

DISCUSSION

In this study, we have developed an *in vitro* model that allows (i) a quantification of the biofilm mass and bacterial viability in naive and induced streptococcal biofilms at different stages of maturity and (ii) a pharmacodynamic evaluation of the activity of antibiotics. The model uses the widely accepted polystyrene support (14, 16, 23, 25, 44, 45), but with important changes from previous studies concerning (i) the medium used for biofilm growth, (ii)

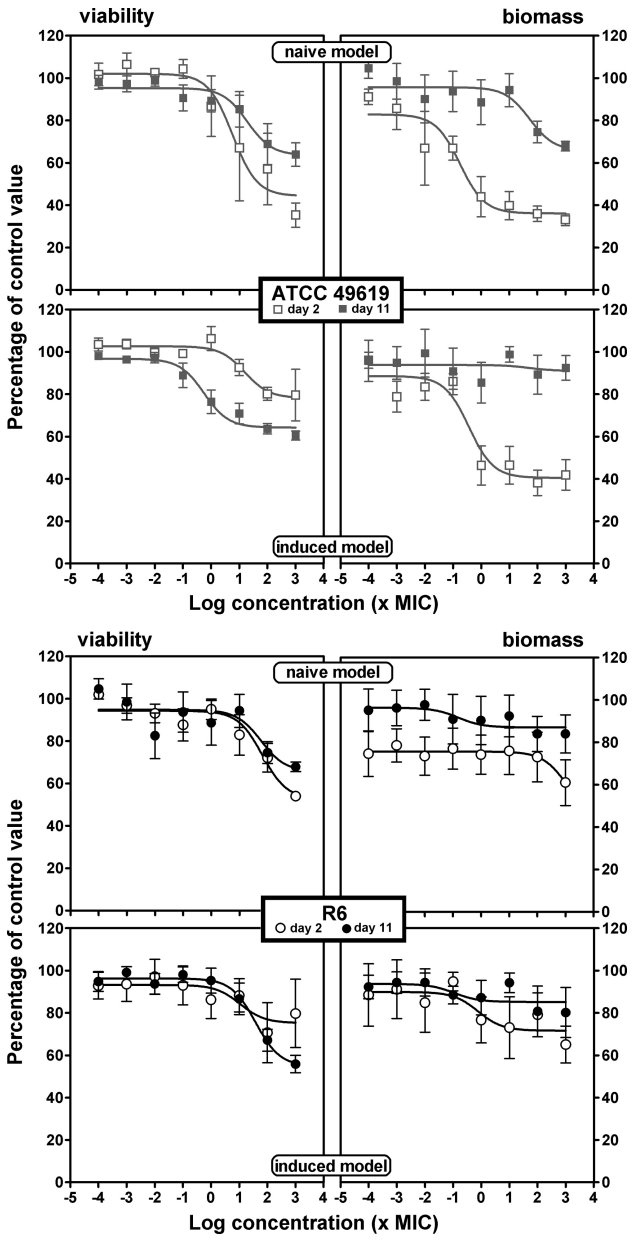

FIG 4 Concentration-response activity of solithromycin against biofilms of ATCC 49619 (top) or R6 (bottom). Two-day-old (open symbols) or 11-day-old (closed symbols) biofilms from the naive model (upper panels for each strain) or the induced model (lower panels for each strain) were incubated with increasing concentrations of solithromycin for 24 h. The ordinate shows the change in viability (measured by the decrease in resorufin fluorescence [left panels]) or in biofilm mass (measured by the decrease in crystal violet absorbance [right panels]) as a percentage of the control value (no antibiotic present). All values are means ± SEM of 4 to 10 independent experiments performed in quadruplicate. When not visible, the error bars are smaller than the size of the symbols. The pertinent pharmacological descriptors of the curves are presented in Tables S1 and S2 in the supplemental material.

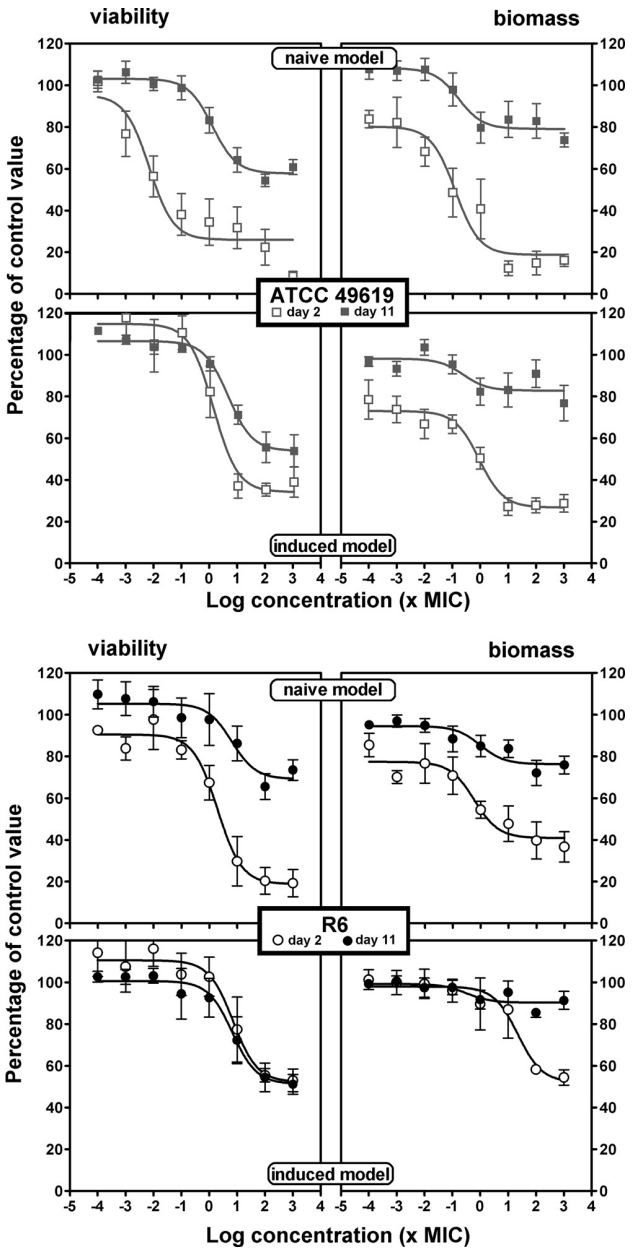

FIG 5 Concentration-response activity of moxifloxacin against biofilms of ATCC 49619 (top) or R6 (bottom). Two-day-old (open symbols) or 11-day-old (closed symbols) biofilms from the naive model (upper panels for each strain) or the induced model (lower panels for each strain) were incubated with increasing concentrations of moxifloxacin for 24 h. The ordinate shows the change in viability (measured by the decrease in resorufin fluorescence [left panels]) or in biofilm mass (measured by the decrease in crystal violet absorbance [right panels]) as a percentage of the control value (no antibiotic present). All values are means ± SEM of 4 to 10 independent experiments performed in quadruplicate. When not visible, the error bars are smaller than the size of the symbols. The pertinent pharmacological descriptors of the curves are presented in Tables S1 and S2 in the supplemental material.

the maturity stages investigated and the impact of adaptation (naive versus induced biofilm), and (iii) the method used to quantify bacterial viability.

With respect to the culture medium, we optimized the conditions of culture not only by adding 2% glucose as recommended previously to increase biofilm formation (20, 45) but also by selecting the medium recommended by CLSI (37) for culture and susceptibility testing of *S. pneumoniae*.

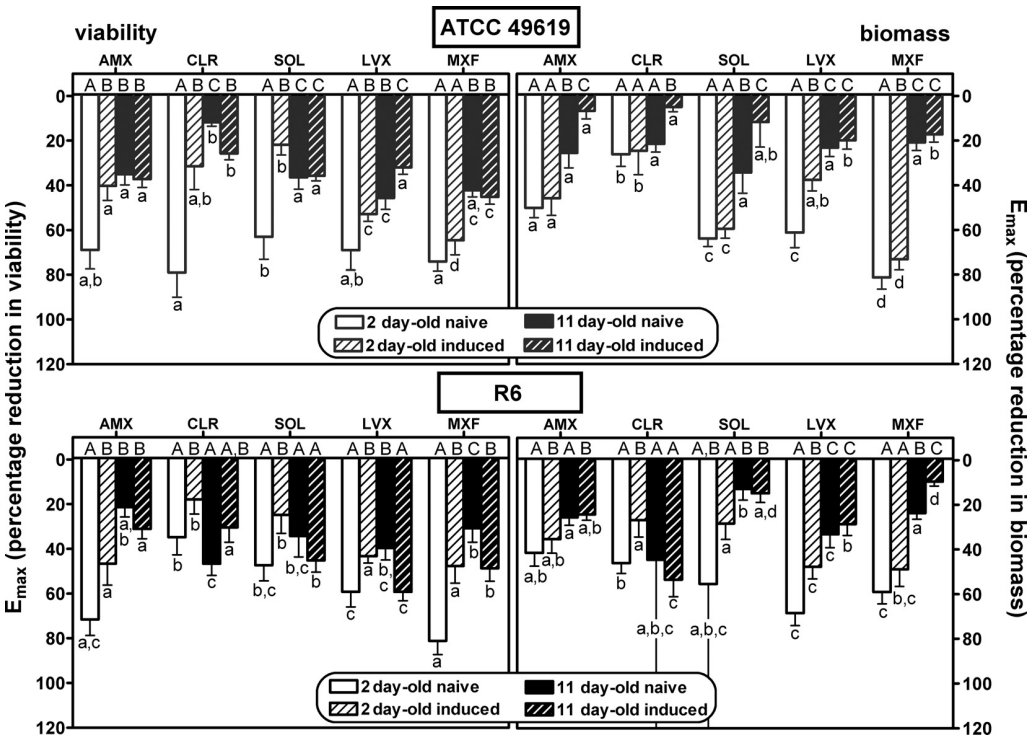

FIG 6 Comparison of antibiotic maximal efficacies (E_{max}) expressed as percentages of reduction in viability (left panels) or biomass (right panels) compared to that in the control (no antibiotic) for 2- and 11-day-old naive and induced biofilms of strain ATCC 49619 (upper panels [gray bars]) or R6 (lower panels [black bars]). AMX, amoxicillin; CLR, clarithromycin; SOL, solithromycin; LVX, levofloxacin; MXF, moxifloxacin. Values were calculated as means ± SEM using the Hill equation of the concentration-response curves presented in Fig. 3 to 5 and Fig. S1 and S2 in the supplemental material. (Also see Tables S1 and S2 in the supplemental material for numerical values.) Statistical analyses were performed by one-way analysis of variance (ANOVA) with Tukey's posttest for multiple comparisons; values with different letters are significantly different from each other ($P < 0.05$). Lowercase letters indicate comparison between antibiotics for each type of biofilm, and capital letters indicate comparison between different types of biofilms for each antibiotic.

With respect to maturity stages, we compared young (2 days) to mature (11 days) biofilms because the latter may represent a more relevant model to study antibiotic activity against persistent forms of infections in deep tissues, where biofilms are suspected to play a role (2, 25, 26). Most of the studies performed so far to evaluate antibiotic activity have indeed used young biofilms only (13, 23, 24), and for those that also considered mature biofilms, only antibiotic effects on the matrix were evaluated (15, 21). We furthermore show that an adaptation process of bacteria is important (*viz.* growth of induced versus naive biofilms). This is probably related to quorum sensing factors, such as the competence-stimulating peptide (CSP), which is produced during biofilm formation and increases bacterial adherence (46).

With respect to quantification of bacterial viability within the biofilm, the method used, namely, resazurin reduction into resorufin, has already been applied to quantify viability of *Staphylococcus aureus* in biofilms (39, 40, 47). We showed here that it can be applied to *S. pneumoniae* biofilms, provided the time after which readings are made is carefully selected to capture the maximal fluorescence signal, which critically depends on both the number of bacteria and the degree of biofilm maturity (since the decrease in the signal can also occur upon too prolonged incubation due to further metabolization in nonfluorescent dihydroresorufin) (42, 48). Because of its proportionality with the number of bacteria, this approach may help in avoiding pitfalls inherent in the other more commonly used method to assess bacterial viability in biofilms, namely, CFU counting after sonication (18, 23, 24, 49, 50). This approach, indeed, was shown to underestimate viability because of the difficulty of quantitatively recovering bacteria from the matrix while at the same time avoiding killing these bacteria (51, 52).

Combining crystal violet staining (for quantification of biomass) with resazurin reduction allows the obtaining of two complementary pieces of information concerning the development of the biofilm, as recently done with *S. aureus* biofilms (47). With those two tools, we show here that the kinetics of biofilm development of *S. pneumoniae* are quite different from those of *S. aureus* with respect to both the rate of attachment and the amount of matrix. Attachment of *S. pneumonaie* is much slower than for *S. aureus* biofilms (47). This may result from a lower expression of adhesins in *S. pneumoniae* than in *S. aureus* (which produces several adhesins, such as the so-called "microbial surface components recognizing adhesive matrix molecules" [MSCRAMMs] or the polysaccharide intercellular adhesin [PIA]) (53). In streptococci, adhesion capacity is described as being highly variable, depending on the phenotype of the colonies (opaque or transparent) and the presence of a capsule (19, 24), yet, phenotypic variation or downregulation of the capsule may occur during biofilm maturation (15, 54). In our hands, no major difference in biofilm formation and maturation was observed between a capsulated strain and an uncapsulated strain, but the demonstration is of limited value, since the strains are not isogenic and may therefore differ by other

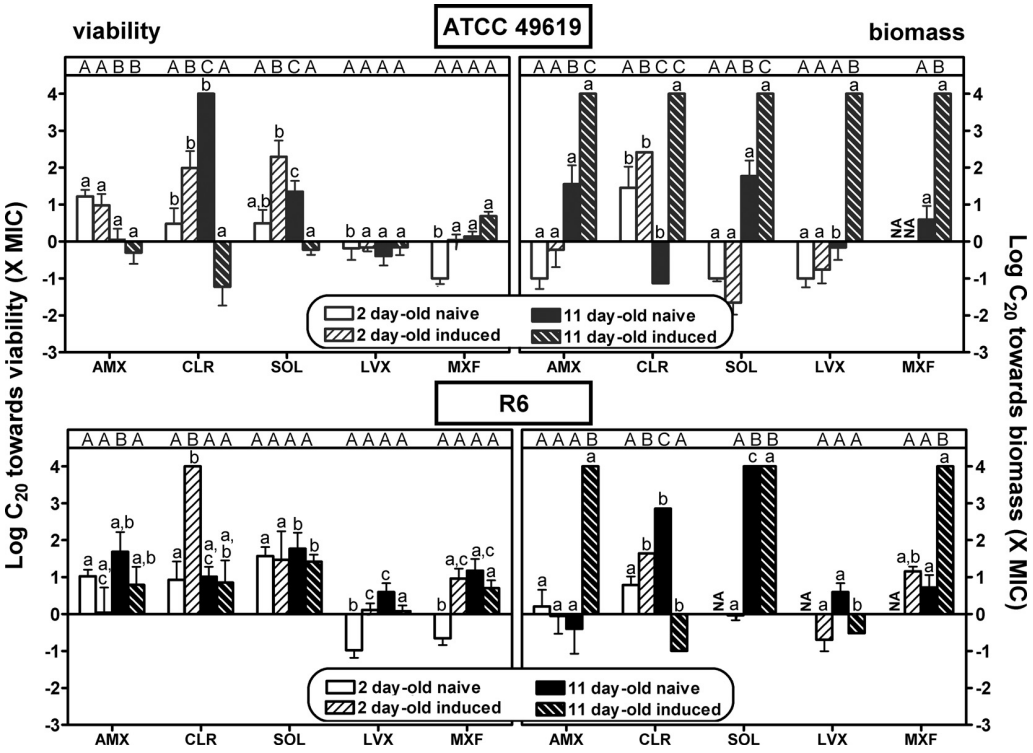

FIG 7 Comparison of antibiotic relative potencies (C_{20}) expressed in multiples of the MIC with respect to viability (left panels) or biomass (right panels) for 2- and 11-day-old naive and induced biofilms of strain ATCC 49619 (upper panels [gray bars]) or R6 (lower panels [black bars]). AMX, amoxicillin; CLR, clarithromycin; SOL, solithromycin; LVX, levofloxacin; MXF, moxifloxacin. Values were calculated as means ± SEM (calculated from the 95% confidence interval band around the curve) using the Hill equation of the concentration-response curves presented in Fig. 3 to 5 and Fig. S1 and S2 in the supplemental material. (Also see Tables S1 and S2 in the supplemental material for numerical values.) Statistical analyses were performed by one-way ANOVA with Tukey's posttest for multiple comparisons; values with different letters are significantly different from each other ($P < 0.05$). Lowercase letters indicate comparison between antibiotics for each type of biofilm, and capital letters indicate comparison between different types of biofilms for each antibiotic. NA, not applicable (a "Top" value of the Hill equation of <80%). When not reached at the maximal value tested, C_{20} values were set at 4 (log scale).

characteristics than the presence of a capsule. With respect to matrix, the higher production observed in streptococci compared to staphylococci could be ascribed to the fact that mature staphylococcal biofilms are characterized by a disassembly phenomenon (mediated by secreted proteases or surfactant-like peptides and regulated by depletion in nutrients in the external environment) (55), which may regulate and limit matrix production. Yet, to our knowledge, this process has never been observed for *S. pneumoniae* biofilms.

We also show here that bacteria released from a preformed biofilm are more prone not only to produce matrix but also to multiply within the biofilm, producing globally thicker structures filled with more bacteria. This suggests that a bacterial adaptation process has taken place during maturation of the naive biofilm. This hypothesis can be placed in correlation with the observation that the protein expression patterns differ between planktonic forms of *S. pneumoniae* and the same strains growing in biofilms with respect to proteins involved in virulence, adhesion, and resistance (49).

Moving now to the quantitative assessment of antibiotic activity, the first and most salient observation is that all responses (for both biomass and bacterial viability) could be analyzed by using the model (Hill equation) commonly used for the analysis of drug-concentration relationships (56) and already applied by us for the study of antibiotic activities against both extracellular and intracellular Gram-positive and Gram-negative bacteria (57, 58), as well as against *S. aureus* biofilms (47). This model offers the possibility to clearly distinguish between two distinct properties of antibiotics, namely, (i) their maximal relative efficacy (using the E_{max} parameter of the Hill equation), which measures the ability of the antibiotic to reduce the biomass or the number of viable bacteria (expressed here as the percentage of the value observed with untreated biofilms) and (ii) their relative potency (the C_{50} or C_{20} parameter), which tells us which drug concentration is needed to obtain a given fraction of its maximal effect, taking into account the type and level of maturation of the biofilm. We therefore suggest that the approach proposed here is more informative than the simple determination of MBIC (minimal biofilm inhibitory concentration) (59) performed in other studies (13, 59) and which gives only a static parameter to describe antibiotic activity against biofilms. As clearly shown here, there is a large divergence between the changes in these two key properties when moving from young to mature and from naive to induced biofilms, with the main consistent changes being related to maximal relative efficacies. Thus, and as for *S. aureus* biofilms (47), antibiotic efficacy for reducing both the bacterial viability and the amount of matrix markedly decreases upon biofilm maturation. However, we see here that antibiotic efficacy is also decreased when biofilms are

TABLE 3 Activities of antibiotics under study on biofilms exposed to concentrations found in epithelial lining fluid

Antibiotic (daily dose, mg)[a]	ELF[b] concn (mg/liter)	Reference	Biofilm model[c]	% viability/matrix loss in:			
				ATCC 49619		R6	
				Viability	Matrix	Viability	Matrix
AMX (3,000)	0.25–1.7	73	2-day-old naive	7–27	47–50	16–44	28–38
			11-day-old naive	30–34	10–19	15–20	25–26
			2-day-old induced	13–32	42–45	36–44	33–35
			11-day-old induced	33–37	5–6	25–30	21–24
			Strong	13–37	5–45	15–44	21–35
CLR (1,000)	4–34	74	2-day-old naive	74–74	24–26	79–80	42–46
			11-day-old naive	42–42	22–22	28–30	6–17
			2-day-old induced	22–30	16–23	17–17	33–35
			11-day-old induced	26–26	5–5	29–30	21–24
			Strong	22–42	5–23	17–30	6–35
SOL (400)	1–7.6	75	2-day-old naive	53–55	64–64	39–46	30–44
			11-day-old naive	32–36	25–33	40–44	13–13
			2-day-old induced	19–22	59–60	24–25	28–28
			11-day-old induced	36–37	8–9	40–44	15–15
			Strong	19–37	8–60	24–44	13–28
LVX (1,000)	2.8–23	76	2-day-old naive	74–74	53–60	63–78	60–67
			11-day-old naive	29–40	22–23	12–26	22–31
			2-day-old induced	37–50	34–37	35–42	40–46
			11-day-old induced	28–31	19–20	42–56	28–29
			Strong	28–50	19–37	12–56	22–46
MXF (400)	3.5–20	77	2-day-old naive	74–74	81–81	79–81	59–59
			11-day-old naive	40–42	21–21	28–30	23–24
			2-day-old induced	61–64	72–73	41–47	36–76
			11-day-old induced	38–44	17–17	44–48	10–10
			Strong	28–64	17–73	28–48	10–76

[a] AMX, amoxicillin; CLR, clarithromycin; SOL, solithromycin; LVX, levofloxacin; MXF, moxifloxacin.
[b] ELF, epithelial lining fluid.
[c] "Strong" represents compilation of data obtained for 11-day-old naive biofilms and 2- or 11-day-old induced biofilms.

formed from trained bacteria (induced biofilms) as opposed to untrained ones (naive biofilms). As a result, and quite interestingly, the effect of antibiotics on bacterial viability was, in most cases, weakened to a similar extent for 11-day-old naive biofilms and 2-day-old induced biofilms compared to 2-day-old naive biofilms. Globally also, antibiotics are least effective against 11-day-old induced biofilms. This may have major implications in terms of chemotherapy, since a reduction in maximal efficacy corresponds to a situation in which a sizeable proportion of bacteria become refractive to the bactericidal effects of antibiotics whatever their concentration in the medium.

The following key observations may also require attention. First, we see that the maximal relative efficacies of antibiotics are somewhat lower (less reduction) when examining the decrease of biomass compared to that of viability for 11-day-old naive biofilms or for 2-day-old induced biofilms and become very low against 11-day-old induced biofilm. This is consistent with the fact that antibiotics primarily act on bacteria and not on the matrix and that destructuration, subsequent to bacterial killing, may become more difficult as the matrix becomes thicker. Second, in contrast to the marked and consistent changes seen for maximal relative efficacies, changes in relative potencies were either minimal or nonsystematic when the effects of age or induction were considered. Detailed analysis here is, however, hampered by the fact that reduction in either viability or biomass was often weak as no C_{50} could be determined. Nevertheless, the data clearly suggest that the effects of maturation and induction on antibiotic activity are related to an apparent reduction in the proportion of reachable targets (bacterial refractory state) and not to a decrease in target apparent affinity (bacterial intrinsic susceptibility). Third, antibiotic maximal efficacies did not differ markedly between the two strains examined, except for clarithromycin and solithromycin, which were more efficient against the capsulated strain in the 2-day-old naive biofilm. This could be related to the capacity of macrolides to downregulate capsule formation (60), since capsule is associated with tolerance to antibiotics (61). Of note also, clarithromycin and solithromycin were less affected than other antibiotics when induced bacteria were used to build up the biofilm. This could be related to the known inhibitory effect of macrolides on quorum sensing (62). Accordingly, macrolides have been shown to increase antibiotic activity on biofilms for *S. aureus* (63).

Our study suffers from at least three limitations. First, we only used two nonisogenic reference strains rather than a collection of clinical isolates obtained from patients with evidence of *in vivo* formation of biofilms. The present study must therefore be viewed as a first pharmacological investigation establishing a model and delineating its main properties with respect to a panel of clinically used antibiotics. The model may now be further explored by using

isogenic strains differing in their expression, their capsule, or virulence factors and by including clinical strains harboring relevant resistance patterns or different serotypes. Second, biofilms were obtained on an artificial support, which is far from the conditions prevailing in the infected body compartments. However, it has been demonstrated (i) that the gene expression profile of *S. pneumoniae* isolates collected from lung tissue resembles that of bacteria grown in biofilm in polystyrene plates (7) and (ii) that biofilm-derived pneumococci (represented here by induced biofilms) possess an enhanced ability to adhere to living support, such as polystyrene coated with epithelial cells (64), which is considered a better model to study biofilm development (65). Adhesins expressed in contact with artificial or viable surfaces may be different (64), however, and therefore may affect biofilm properties. Also, there are reports suggesting that the ability to form early biofilms *in vitro* does not reflect virulence potential *in vivo* (17) and does not necessarily correlate with the clinical presentation of pneumococcal disease (22). Finally, bacteria were exposed to constant concentrations of antibiotics, and records were made at only one fixed time point. These conditions do not mimic the pharmacokinetic profile of the drugs in the lung and do not inform us about differences in progression of the effects seen. The model, therefore, must be viewed as a first approach open for improvement.

With these limitations, however, our work can be examined in a more clinical perspective, considering the range of antibiotic concentrations reached in human bronchoalveolar lavage fluid (Table 3). Among the drugs investigated, fluoroquinolones and clarithromycin are more effective against 2-day-old naive biofilms, causing an approximately 70 to 80% reduction in viability. Clarithromycin activity, however, is severely hampered today by high resistance rates around the globe (66–68). Against mature or induced biofilms and with the range of clinically relevant concentrations, fluoroquinolones reduce viability more than amoxicillin and macrolides/ketolides, highlighting a potentially greater activity for the first class of drugs. This is consistent with a previous, noncomparative study showing that moxifloxacin, at concentrations that can be achieved in the bronchial mucosa during therapy, was able to inhibit biofilm synthesis and induce slime disruption (23). However, differences among classes are not major, and other considerations, like resistance rates or patients' susceptibility to undesirable effects or drug interactions, are important determinants to take into account in antibiotic selection. Moxifloxacin and solithromycin may thus offer an advantage over the other molecules within their pharmacological class since both molecules are less prone to select resistance than others in their respective class (31, 69). Moreover, moxifloxacin MICs have remained stable over the last 10 years, despite extensive usage (70, 71), and preclinical studies show that solithromycin is barely affected by mechanisms conferring resistance to other macrolides (31, 72).

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AAC1858-13

Antibiotic activity against naive and induced *Streptococcus pneumoniae* biofilms in an *in vitro* pharmacodynamic model

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SUPPLEMENTARY MATERIAL

Figure S1

Concentration-response activity of clarithromycin against biofilms of ATCC 49619 (top) or R6 (bottom). Two-day- (open symbols) or 11-day- (closed symbols) old biofilms from the naive model (upper panels for each strain) or the induced model (lower panels for each strain) were incubated with increasing concentrations of clarithromycin for 24h. The ordinate shows the change in viability (measured by the decrease in resorufin fluorescence; left panels) or in biofilm mass (measured by the decrease in crystal violet absorbance; right panels) in percentage of the control value (no antibiotic present). All values are means ± SEM of 4-10 independent experiments performed in quadruplicates (when not visible, the bars are smaller than the size of the symbols). The pertinent pharmacological descriptors of the curves are presented in Tables S1-S2.

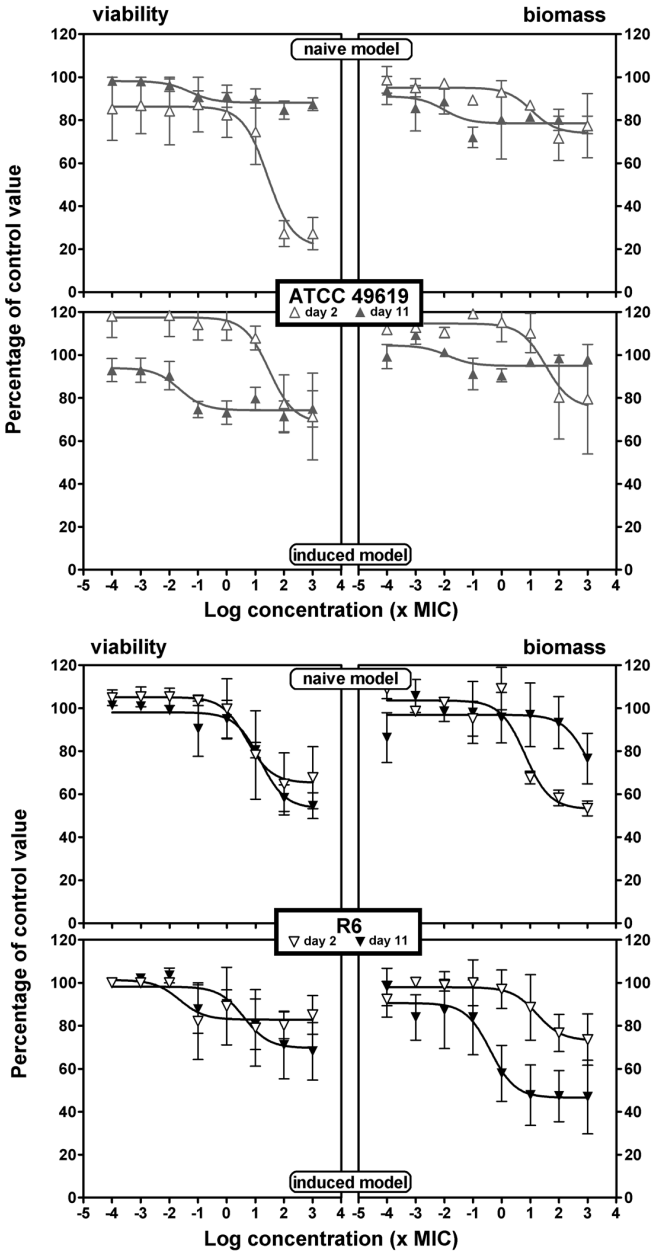
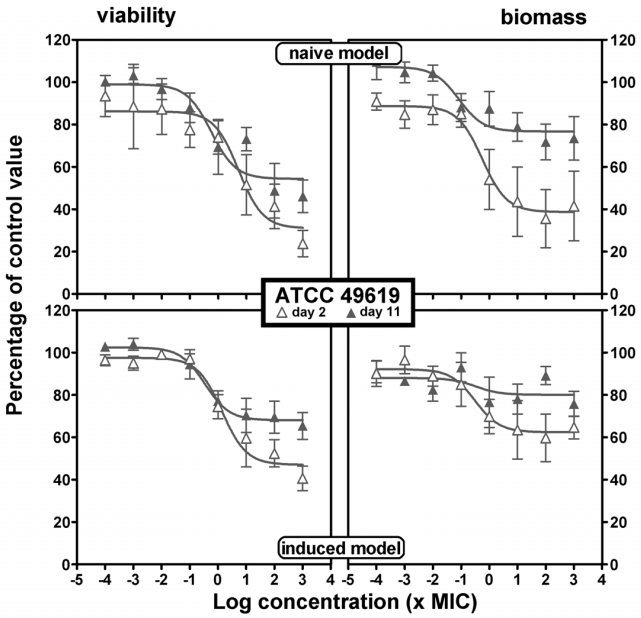


Figure S2

Concentration-response activity of levofloxacin against biofilms of ATCC 49619 (top) or R6 (bottom). Two-day- (open symbols) or 11-day- (closed symbols) old biofilms from the naive model (upper panels for each strain) or the induced model (lower panels for each strain) were incubated with increasing concentrations of levofloxacin for 24h. The ordinate shows the change in viability (measured by the decrease in resorufin fluorescence; left panels) or in biofilm mass (measured by the decrease in crystal violet absorbance; right panels) in percentage of the control value (no antibiotic present). All values are means ± SEM of 4-10 independent experiments performed in quadruplicates (when not visible, the bars are smaller than the size of the symbols). The pertinent pharmacological descriptors of the curves are presented in Tables S1-S2.



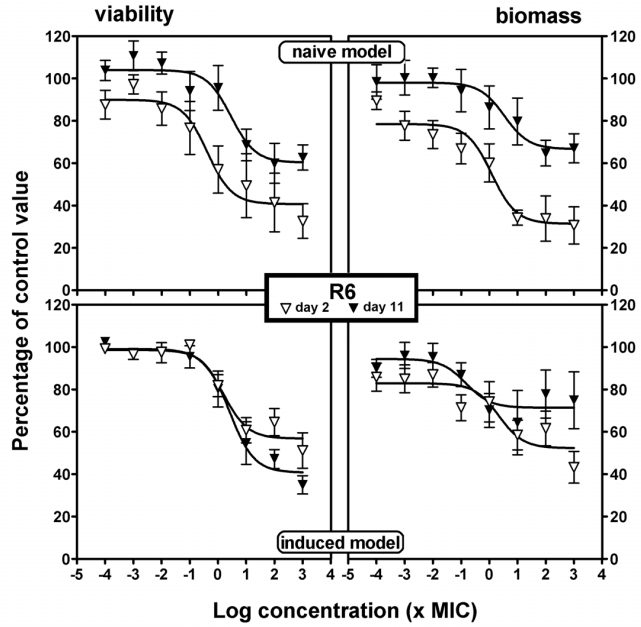


Table S1. Pertinent regression parameters[a] (with 95% confidence intervals) and statistical analysis[c] for strain ATCC 49619

		Effect on viability within the matrix					Effect on biofilm thickness				
		E_{max}[b]	Concentration yielding specified effect				E_{max}[b]	Concentration yielding specified effect			
		% loss of viability	20% reduction		50% reduction		% loss of matrix	20% reduction		50% reduction	
AB	Biofilm model	(CI at 95%)	(in X MIC)	(in mg/L)	(in X MIC)	R^2	(CI at 95%)	(in X MIC)	(in mg/L)	(in X MIC)	R^2
AMX	2 days; naive	68.89 (51.80 to 85.98) / A[c]	16.5 (9.7 to 23.5) / A[c]	1.1	111.3	0.648	50.13 (40.71 to 59.55) / A	0.1(-11.8 to 12) / A	< 0.1	101.3	0.430
	2 days; induced	40.21 (26.96 to 53.46) / B	7.6 (-3.4 to 22.5) / A	0.5	$>10^4$	0.589	45.83 (29.74 to 61.92) / A	0.2 (-27.8 to 29) / A	< 0.1	$>10^4$	0.413
	11 days; naive	35.01 (25.31 to 44.71) / B	0.9 (12.23 to 14.43) / B	0.1	$>10^4$	0.500	25.56 (11.78 to 39.34) / B	35.8 (-2.8 to 74.4) / B	2.3	$>10^4$	0.206
	11 days; induced	37.17 (29.57 to 44.77) / B	0.5 (-12.23 to 13.23) / B	0.1	$>10^4$	0.626	6.72 (-0.78 to 14.22) / C	$> 10^4$ / C	> 640	$>10^4$	0.147
CLR	2 days; naive	79.01 (67.91 to 90.11) / A	2.9 (-20.1 to 26.13) / A	< 0.1	33.5	0.648	26.08 (14.72 to 37.44) / A	28.2 (-14.4 to 70.8) / A	60.9	$>10^4$	0.396
	2 days; induced	31.46 (21.04 to 41.88) / B	98.6 (70.6 to 124.9) / B	3.2	$>10^4$	0.552	24.59 (1.54 to 47.64) / A	260 (88.2 to 431.2) / B	8.3	$>10^4$	0.553
	11 days; naive	11.87 (10.27 to 13.47) / C	$> 10^4$ / C	> 320	$>10^4$	0.427	21.50 (13.76 to 29.24) / A	0.1 / C	< 0.1	$>10^4$	0.137
	11 days; induced	25.74 (22.9 to 28.58)/ B	0.06 (-33.5 to 33.5) / A	< 0.1	$>10^4$	0.367	5.00 (0.47 to 9.53) / B	$> 10^4$ / C	> 320	$>10^4$	0.325
SOL	2 days; naive	63.03 (50.63 to 75.43) / A	3.5 (-15.25 to 21.4) / A	< 0.1	9.2	0.746	63.88 (56.51 to 71.25) / A	0.1 (-4 to 4.3) / A	< 0.1	0.4	0.718
	2 days; induced	21.86 (12.71 to 31.01) / B	192.5 (170.3 to 220) / B	- 1.5	$>10^4$	0.499	59.48 (50.78 to 68.18) / A	0.1 (-15.7 to 15.7) / A	< 0.1	1.4	0.634
	11 days; naive	36.4 (25.56 to 47.24) / C	17.7 (9.3 to 34.9) / C	- 0.1	$>10^4$	0.491	34.26 (15.41 to 53.11) / B	58.6 (33.9 to 83.3) / B	0.5	$>10^4$	0.277
	11 days; induced	35.74 (31.20 to 40.28) / C	0.6 (-5 to 6.3) / A	< 0.1	$>10^4$	0.733	11.64 (0.36 to 22.52) / C	$> 10^4$ / C	> 80	$>10^4$	0.050
LVX	2 days; naive	69.06 (51.60 to 86.52) / A	0.7 (-14.8 to 16.1) / A	0.7	10.0	0.434	61.22 (47.73 to 74.71) / A	0.1 (-11.5 to 11.7) / A	0.1	2.0	0.408
	2 days; induced	52.90 (46.22 to 59.58) / B	0.7 (-4 to 5.4) / A	0.7	21.6	0.829	37.56 (27.42 to 47.70) / B	0.2 (21.4 to 21.7) / A	0.2	$>10^4$	0.334
	11 days; naive	45.63 (35.23 to 56.03) / B	0.4 (-10.4 to 11.2) / A	0.4	$>10^4$	0.495	23.23 (15.46 to 31.00) / C	0.7 (-16.5 to 18) / A	0.7	$>10^4$	0.378
	11 days; induced	31.92 (25.59 to 38.25) / C	0.7 (-7.8 to 9.2) / A	0.7	$>10^4$	0.598	19.93 (11.86 to 28.00) / C	$> 10^4$ / B	$> 10^4$	$>10^4$	0.073
MXF	2 days; naive	74.07 (69.76 to 78.38) / A	0.1 (-6 to 6.3) / A	< 0.1	0.1	0.569	81.25 (70.63 to 91.87) / A	NA[d]	NA[d]	0.1	0.620
	2 days; induced	64.62 (58.22 to 71) / A	1.0 (-5 to 7.2) / A	0.1	5.9	0.765	73.19 (63.55 to 82.83) / A	NA[d]	NA[d]	0.9	0.655
	11 days; naive	42.18 (39.19 to 45.17) / B	1.3 (-4.2 to 7) / A	0.2	$>10^4$	0.801	20.87 (13.59 to 28.15) / B	3.9 (-17.2 to 25) / A	0.5	$>10^4$	0.454
	11 days; induced	45.14 (41.85 to 48.42) / B	4.7 (-0.47 to 10) / A	0.6	$>10^4$	0.847	17.21 (10.37 to 24.05) / B	$> 10^4$ / B	> 1250	$>10^4$	0.193

[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 post test for multiple comparisons between different types of biofilms for each drug, values with different letters are significantly different from each other ($P<0.05$); see figures 6 and 7 for comparisons between antibiotics for each type of biofilm.

[d] not applicable (TOP of the Hill equation close to 80%)

Table S2. Pertinent regression parameters[a] (with 95% confidence intervals) and statistical analysis[c] for strain R6

		Effect on viability within the matrix					Effect on biofilm thickness				
		E_{max}[b]	Concentration yielding specified effect				E_{max}[b]	Concentration yielding specified effect			
		% loss of viability	20% reduction		50% reduction		% loss of matrix	20% reduction		50% reduction	
AB	Biofilm model	(I at 95%)	(in X MIC)	(in mg/L)	(in X MIC)	R^2	(CI at 95%)	(in X MIC)	(in mg/L)	(in X MIC)	R^2
AMX	2 days; naive	71.60 (57.16 to 86.04) / A[c]	11.1 (3.6 to 17.5) / A [c]	0.4	79.08	0.748	41.45 (29.26 to 53.64) / A	1.6 (-27 to 30.2) / A	0.1	$>10^4$	0.294
	2 days ;induced	41.11 (21.52 to 60.70) / B	1.1 (70.1 to 72.3) / A	0.1	$>10^4$	0.258	35.41 (22.70 to 48.12) / A	0.6 (39.4 to 31.1) / A	< 0.1	$>10^4$	0.417
	11 days; naive	21.3 (12.38 to 30.22) / B	48.3 (4.1 to 92.4)/ B	1.5	$>10^4$	0.298	25.36 (18.23 to 32.49) / B	0.4 (-88.2 to 89) / A	< 0.1	$>10^4$	0.189
	11 days; induced	31.04 (21.62 to 40.46) / B	3.5 (-23.9 to 36.3) / A	0.1	$>10^4$	0.589	24.24 (18.81 to 29.67) / B	$> 10^4$ / B	> 320	$>10^4$	0.826
CLR	2 days; naive	34.75 (26.87 to 42.63) / A	8.5 (-21.9 to 39) / A	0.1	$>10^4$	0.503	46.03 (38.48 to 53.58) / A	5.9 (-4.7 to 16.9) / A	0.4	$>10^4$	0.881
	2 days; induced	17.82 (11.26 to 24.38) / B	$> 10^4$ / B	> 640	$>10^4$	0.472	26.84 (11.01 to 42.67) / B	44.0 (-16.8 to 104.6) / B	2.8	$>10^4$	0.332
	11 days; naive	46.59 (41.35 to 51.83) / A	9.9 (-2.2 to 22.8) / A	0.6	$>10^4$	0.767	44.59 (-304.62 to 393.80) / A	715.1 / C	45.6	$>10^4$	0.132
	11 days; induced	30.34 (23.63 to 37) / A,B	7.1 (-39.3 to 53.6) / A	0.5	$>10^4$	0.411	53.50 (37.71 to 62.29) / A	0.1 (-43.4 to 43.6) / A	< 0.1	1.17	0.478
SOL	2 days ;naive	47.29 (33.04 to 61.54) / A	33.8 (27.5 to 46.8) / A	0.1	$>10^4$	0.572	55.40 (-127.4 to 171.8) /A,B	NA[d]	NA[d]	5307.13	0.043
	2 days; induced	24.78 (41.50 to 8.06) / B	29.8 (-119.6 to 178.6) / A	0.1	$>10^4$	0.105	28.38 (21.27 to 35.5) / A	0.9 (0.5 to 2.3) / A	< 0.1	$>10^4$	0.099
	11 days; naive	34.26 (15.41 to 53.11) / A	59.8 (33 to 84.4) / A	0.2	$>10^4$	0.277	12.93 (7.92 to 17.94) / B	$> 10^4$ / B	> 40	$>10^4$	0.042
	11 days; induced	45.18 (34.74 to 55.62) / A	24.6 (18.6 to 33.6) / A	0.1	$>10^4$	0.688	14.84 (10.77 to 18.91) / B	$> 10^4$ / B	> 40	$>10^4$	0.052
LVX	2 days; naive	59.24 (45.59 to 72.89) / A	0.1 (-9.8 to 10) / A	0.1	1.77	0.371	68.52 (62.93 to 74.1) / A	NA[d]	NA[d]	1.86	0.511
	2 days; induced	43.21 (36.62 to 49.80) / B	1.3 (-6 to 8.6) / A	0.7	$>10^4$	0.800	47.72 (42.2 to 53.24) / B	0.2 (-17.1 to 17.5) / A	0.1	$>10^4$	0.306
	11 days; naive	39.64 (28.75 to 50.53) / B	3.5 (-6.1 to 13.9) / A	1.8	$>10^4$	0.530	33.28 (27.37 to 39.2) / C	4.6 (-6.1 to 13.9) / A	2.3	$>10^4$	0.328
	11 days; induced	59.21 (51.05 to 67.37) / A	1.2 (-4.9 to 7.3) / A	0.6	13.49	0.808	28.63 (23.58 to 33.68) / C	0.3 (-63.3 to 63.9) / A	0.2	$>10^4$	0.212
MXF	2 days; naive	81.18 (68.82 to 95.54) / A	0.3 (-7.5 to 7.9) / A	< 0.1	2.56	0.694	59.06 (48.27 to 69.85) / A	NA [d]	NA [d]	1.78	0.513
	2 days; induced	47.73 (32.28 to 63.18) / B	7.9 (-1.9 to 20.1) / A	0.5	$>10^4$	0.638	47.88 (32.02 to 63.74) / A	14.5 (2.9 to 29.9) / A	0.5	$>10^4$	0.609
	11 days; naive	30.82 (18.16 to 43.48) / C	14.9 (0.7 to 29) / A	1.0	$>10^4$	0.435	23.67 (17.95 to 29.39) / B	5.2 (-12.5 to 22.9) / A	0.3	$>10^4$	0.365
	11 days; induced	48.73 (37.21 to 60.25) / B	4.8 (-3.3 to 13.3) / A	0.3	$>10^4$	0.636	9.66 (5.64 to 13.68) / C	$> 10^4$ / B	> 640	$>10^4$	0.282

[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 post test for multiple comparisons between different types of biofilms for each drug, values with different letters are significantly different from each other ($P<0.05$); see figures 6 and 7 for comparisons between antibiotics for each type of biofilm.

[d] not applicable (TOP of the hill equation close to 80 %)

4. Bronchodilators-mediated increase of the antibiotic activity against pneumococcal biofilms

The previous experimental study has shown that antibiotic activity is highly dependent on the biofilm thickness (old maturity stages and induction of matrix production) and globally higher on bacterial survival than on biomass. Several compounds such as antiseptics or ions (e.g. fluoride) have already been described in the literature as presenting anti-biofilm properties by targeting the matrix (Lellouche *et al.*, 2012). Because of their chronic respiratory symptoms and frequent co-morbidities, COPD patients are constantly polymedicated, whether in absence or presence of acute exacerbations episodes.

Therefore, we decided, in a next step, to study the potential benefits that non-antibiotic drugs may have by decreasing the matrix thickness or preventing its production and so, improving the antibiotic activity on which biofilms were exposed afterwards.

In this context of AECB, we decided to investigate the impact of biofilm growth in the presence of salbutamol and/or ipratropium, two different types of bronchodilators on the activity of two fluoroquinolones and one fluoroketolide, actually in phase 3 of clinical development. Fortunately, highly significant improvement of their activity on biomass and survival of sessile bacteria, mediated by bronchodilators, could be observed. These results and underlying mechanisms are described in the following original paper (currently submitted).

AUTHORS' CONTRIBUTIONS:

1. Conceived and designed experiments: NMV;
2. Performed experiments: NMV, GGM;
3. Analyzed data: NMV, PMT, FVB;
4. Wrote the paper: NMV, PMT, FVB.

Bronchodilators improve antibiotic activity against pneumococcal biofilms by altering matrix.

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Key points (40 word): Ipratropium (trough biofilm disassembly) and albuterol (through stimulation of neuraminidase activity) improve the activity of moxifloxacin and solithromycin against pneumococcal biofilms. Bronchodilators may contribute to bacterial clearance from respiratory airways. In patients with acute bacterial exacerbations of chronic bronchitis.

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Running head: Bronchodilators, antibiotics and biofilms

Paper additional metrics:
Tables: 2 / Figures: 8
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References: 40
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Methods word count: 484
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ABSTRACT

Background. Due to matrix barrier effects, antibiotics are poorly active against bacteria in biofilms formed by *Streptococcus pneumoniae* isolated from patients with acute exacerbations of chronic bronchitis (AECB). We aimed at determining whether bronchodilators modulate pneumococcal biofilms development *in vitro* and improve antibiotic activity.

Methods. 47 strains of *S. pneumoniae* from hospitalized AECB patients were screened for production of naive and induced biofilms. Using one typical clinical isolate and two reference strains, we tested for the ability of ipratropium and/or albuterol, added at therapeutic concentrations during biofilm formation, to (i) modulate biofilm development; (ii) improve the activity of amoxicillin, clarithromycin, moxifloxacin, or solithromycin (a novel ketolide) for bacterial killing (resazurin reduction) and decrease of biomass (crystal violet staining).

Results. All clinical isolates produced naive and induced biofilms, but with reduced thickness if collected from patients having received muscarinic antagonists. Growing biofilms in the presence of ipratropium caused (i) a reduced biomass formation and faster disassembly associated with free sialic acid release in supernatant; (ii) a marked improvement of activity of solithromycin and moxifloxacin, with bacterial killing and biomass reduction up to 100% for the latter. Albuterol stimulated neuraminidase activity and, thereby, improved antibiotic killing activity (reversed by zanamivir, an inhibitor of pneumococcal neuraminidase A) with only modest biomass reduction.

Conclusions. Trough biofilm disassembly (ipratropium) and stimulation of neuraminidase activity (albuterol), bronchodilators improve the activity of solithromycin and moxifloxacin against pneumococcal biofilms. This may rationalize the use of bronchodilators in patients with AECB for more effective bacterial clearance from respiratory airways.

Abstract word count: 250 (max. 250)

Keywords (5): *Streptococcus pneumoniae*, ipratropium, albuterol, moxifloxacin, solithromycin

INTRODUCTION

Streptococcus pneumoniae is a major pathogen associated with acute exacerbations of chronic bronchitis (AECB) [1, 2]. Its capacity to form biofilms favors persistence in the airways [3] and may contribute to chronic colonization [4, 5] and relapses [6]. Isolates of *S. pneumoniae* obtained from patients suffering from AECB are also high biofilm producers *in vitro* [7]. Within biofilms, bacteria are embedded in an extracellular polymeric matrix that creates a diffusion barrier to antibiotics, thereby contributing to a poor response to treatment [4, 8-10]. Destructuring biofilm matrix is, therefore, an appealing strategy for improving antibiotic effectiveness [11-13].

Patients suffering from chronic obstructive pulmonary disease (COPD) usually receive bronchodilators as first-line therapy [14]. We therefore wondered whether these drugs could modify the development and susceptibility to antibiotics of naive and induced biofilms (reflecting primo infection and secondary colonization, respectively [15]). We collected clinical isolates from the sputum of patients with a confirmed diagnosis of AECB requiring hospitalization, and examined their capacity to produce biofilm in previously validated *in vitro* pharmacodynamic models [15]. Having observed no significant differences in biofilm formation between these clinical isolates and two reference strains, we focused on two reference strains and one clinical isolate to investigate the influence exerted by ipratropium and albuterol (known in Europe as ALBbutamol) on biofilm growth (these two drugs being selected as typical representatives of the main bronchodilator pharmacological classes for COPD patients). We also examined whether these bronchodilators modulate antibiotic activity against biofilms, selecting first amoxicillin and clarithromycin, i.e. two representatives of β-lactams and macrolides, respectively, and commonly recommended in patients suffering from AECB [14], and then moving to moxifloxacin (because of its reported higher efficacy in the treatment of AECB compared to other antibiotics [16, 17]), and solithromycin (a fluoroketolide currently in clinical development for the treatment of low respiratory tract infections [18] and active against macrolide-resistant strains [19]). The latter two antibiotics have also shown to be the most effective within their respective class in the model of biofilm used here [15].

METHODS

AECB Patients, pneumococcal strains and antibiotic susceptibility testing

Forty-seven *S. pneumoniae* isolates were collected from patients with (i) confirmed AECB diagnosis (Anthonisen's criteria [20]), (ii) specimen fulfilling the interpretive criteria of lower respiratory tract origin [21], (iii) hospitalization, and (iv) anamnestic confirmation of recent/current bronchodilator use. Data were thereafter anonymised. Minimal inhibitory concentrations (MICs) were determined by microdilution according to the Clinical Laboratory Standards Institute recommendations [22].

Biofilm models

Naive and induced biofilms were obtained as previously described [15]. Biofilms were cultivated in control medium or in medium supplemented with ipratropium (1.45 mg/L), albuterol (7.25 mg/L), zanamivir (250 mg/L), or their combination (concentrations mimic those expected to be measured in the epithelium lining fluid of patients upon single administration of the inhaled form of ipratropium or albuterol, or inhibiting the pneumococcal neuraminidase A for zanamivir; see details in Supplementary Text 1).

Biomass, bacterial viability quantifications and antibiotics activity towards biofilms

Biomass was measured by crystal violet staining, and bacterial viability by the reduction of resazurin to fluorescent resorufin [15]. Antibiotic activity was measured exactly as previously described using 2-days and 11-days biofilms with antibiotics concentrations ranging from 10^{-4} to 10^{3} x their MIC in broth. Data were used to fit a Hill equation (sigmoid) as a function of the antibiotic concentration to determine their relative maximal efficacies (E_{max}) and potencies (concentration yielding a 50 % of E_{max} [C_{50}]), two key pharmacological descriptors of activity against biofilms [15].

Free sialic acid assay in biofilm supernatant

Free sialic was extracted from the supernatant and its concentration determined by both HPLC-MS and enzymatic (Sialic acid quantification kit – [cat. no. SIALICQ] Sigma-Aldrich, St-Louis, MO) assays with linear correlation between both methods (R^2= 0.966; slope [enzymatic/HPLC-MS] = 0.841±0.059; see further details in Supplementary Text 2.

Sialic acid release from *S. pneumoniae* by bacterial neuraminidase

Bacteria were incubated (3h; 37°C) in phosphate buffered ALBine pH 7.4 with or without purified *Arthrobacter ureafaciens* $\alpha(2\rightarrow3,6,8,9)$ neuraminidase A (Sigma-Aldrich, St Louis, MO) in the presence or absence of albuterol, zanamivir, or their combination. Released sialic acid was then quantified by the enzymatic assay described above.

Pharmacological agents

Albuterol and ipratropium were the solutions for nebulization distributed for clinical use in Belgium (Ventolin®[ALBbutamol], GlaxoSmithKline, Genval; Atrovent®, Boehringer Ingelheim, Ingelheim am Rhein, Germany). Zanamivir was purchased

from Sigma-Aldrich (St Louis, MO). Amoxicillin was obtained as the branded product for human parenteral use complying with the prescriptions of the European Pharmacopoeia (>90% purity) and distributed for clinical use 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), respectively.

Curve fitting, correlations and statistical analyses

These were made with GraphPad Prism® 4.03 and GraphaPad Instat® 3.10 (GraphPad software, San Diego, CA) or JMP® 10.0.2 (SAS Institute Inc., Cary, NC).

RESULTS

Patient's main characteristics

Table 1 shows the patients main characteristics. The majority of patients were almost equally distributed within the 55-64, 65-74 and ≥75 years groups. Comorbidities (diabetes, lung cancer [primitive or with metastases], alcoholism, psychiatric disorders, hypertension) were frequent. Most patients were men, lived at home prior hospitalization, and about 60% were active smokers. Incidences of obstruction severity according to GOLD scores [14] were almost equally distributed between low (1-2) and high (3-4) levels.

Correlations between medications and severity factors

Table 2 shows that β-2 agonists, muscarinic antagonists, long-acting bronchodilators, and inhaled corticoids use prior to hospitalization were significantly associated with a higher COPD severity (GOLD scores 3-4) upon admission and a prolonged hospitalization (>10 days). Short-acting bronchodilators intake was associated with high obstruction severity.

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

Naive biofilms were generated from all clinical strains and biomass quantified after 10 days of culture in control medium. Results were stratified according to (i) COPD severity upon admission (using GOLD scores [14]) and (ii) patients' bronchodilator medication prior hospitalization. No significant association between biomass amounts and GOLD scores was found but strains collected from patients having received only muscarinic antagonists produced less biomass than strains coming from patients receiving no treatment or other bronchodilator(s) (one-way ANOVA with Tukey's post-test, $p<0.05$; unshown data).

Influence of bronchodilators on biofilm formation from selected strains

Because no correlation was observed in the collection of clinical strains between the rate of biofilm formation and the severity of patients' respiratory obstruction, we decided to investigate the influence of bronchodilators on the development of biofilms by selecting one clinical isolate (N6 [capsulated; serotype 35B]) originating from a typical COPD patient (respiratory tract colonization with both *S. pneumoniae* and *H. influenzae*; severe respiratory obstruction [GOLD score 3]; two frequent co-morbidities [hypertension and psychiatric disorders; [23-25]]; deep tobacco addiction; pre-admission treatment: fenoterol/ipratropium). In parallel, we selected two reference strains, namely the ATCC49619 (capsulated, serotype 19F; used as international reference for pneumococcal susceptibility testing [22] and the R6 (uncapsulated; often used for *in vitro* studies of pneumococcal biofilm architecture [26] and the implication of neuraminidase A in biofilm formation [27]).

As previously shown [15], under control conditions, biofilm formation was more important in the induced than in the naive model for all 3 strains (Figure 1). Of note, for the clinical strain, the intense growth 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 [28]. For the

naive model, addition of ipratropium did not affect biomass increase up to day 8 but was thereafter associated with an almost complete loss of biomass at day 11 for all 3 strains. In the induced model, ipratropium (i) had a similar effect than in the naïve model for the reference R6 strain, (ii) caused a marked inhibition of biomass formation for the reference ATCC49619 strain at day 7 and a loss of biomass thereafter; (iii) impaired the formation of biomass at day 9 and an almost complete loss of this biomass at day 11 for the clinical N6 strain. In sharp contrast, albuterol was without marked effect on biomass overtime compared to control. Addition of both drugs caused intermediate effects.

Antibiotics activity on biofilms grown in the presence of albuterol combined with ipratropium

We first examined whether growing biofilms in the presence of the combination of albuterol and ipratropium modified the activity of antibiotics with respect to both bacterial viability and biomass using the ATCC49619 reference strain and both 2-days naive and 11-days induced biofilms (Figure 2). For amoxicillin and clarithromycin, only modest effects were observed with 2-days naive biofilms and no effect with 11-days induced biofilms. Conversely, moxifloxacin and solithromycin activities were markedly enhanced for maximal efficacy (E_{max}) and potency (C_{50} or C_{25}, as appropriate), especially if considering bacterial viability. Based on this first set of observations, only moxifloxacin and solithromycin were used for further studies.

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

We first checked that ipratropium, albuterol or zanamivir (at concentrations up to 4, 8, and 8 mg/L, respectively) did change the MICs of the antibiotics. In the 11-days biofilm model (Figure 3A), moxifloxacin completely suppressed the viability signal (reduction of resazurin) when the biofilm was grown in the presence of ipratropium, as compared to only 50 % in control biofilms. This, interestingly enough, was even better than what was observed for the highly susceptible 2-days naive biofilms in non-supplemented medium (control; compare to Figure 2). Similar effects on biomass were observed for the reference ATCC49619 and R6 strains, and to a lesser extent for the clinical strain N6. Moreover, moxifloxacin potency (C_{50}) was also improved by ipratropium. For solithromycin (Figure 3B), growing biofilms in the presence of ipratropium exerted an effect that was qualitatively similar to what had been observed for moxifloxacin but quantitatively less marked.

In contrast, growing biofilms in the presence of albuterol alone was without marked effect on moxifloxacin or solithromycin activity against the 11-days induced biofilm, except for viability with the reference R6 strain (see Figures 3A and B). However, when tested in the 2-days induced model, albuterol improved moxifloxacin and solithromycin killing activities against biofilms formed by the two reference strains, and this effect was blocked by zanamivir (Figure 4; see data the 2-days naive model in Supplementary Figures 1-6). Albuterol did not antagonize the effects of ipratropium on antibiotic activity (see Supplementary Figures 1-6 of the online data supplement).

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

Since biofilm cohesion depends on sialic acid-mediated intra-bacterial bounds [29], we checked whether the biomass decrease observed with biofilms grown in the presence of ipratropium and their increased susceptibility to antibiotics was associated with the release of free sialic acid in the medium. Figure 5 (left panel) shows that sialic acid was indeed released in larger amounts when biofilms had been grown in the presence of ipratropium for the two reference strains (ATCC49619 and R6) compared to control conditions. For the same strains, this sialic acid release was associated with a marked viability loss after incubation with antibiotics (see middle [moxifloxacin] and right [solithromycin] panels; the effect appears modest for solithromycin because of the lower global activity of this antibiotic towards mature biofilms noted in Figure 2). For the clinical strain N6, little sialic acid release was detected at day 11 because the complete biofilm destructuration had already been achieved earlier (see Figure 1) and was accompanied by free sialic acid release mainly occurring between day 2 and 7 (data not shown). Therefore, no association could be established between this release and biomass or loss of viability.

Since we had observed that zanamivir completely abolished the effect of albuterol on antibiotic efficacy (Figure 4), we examined in more details the changes in sialic acid release in biofilms exposed to albuterol with and without zanamivir. Focusing first on 11-days biofilms (to compare with ipratropium), no clear correlation was seen between free sialic acid levels and biomass (Figure 6A), partly because of a high variability between strains. These were, therefore, examined individually for both 2-days and 11-days old and for naive and induced biofilms. Figure 6B shows that exposure to albuterol systematically increased sialic acid release for biofilms formed by the reference strains (ATCC49619 and R6) and that this increase was suppressed by zanamivir. For the clinical N6 strain, albuterol only increased the sialic acid release for the 2-days old induced biofilm, and zanamivir exerted no or little effect.

Modulation of bacterial neuraminidase activity by albuterol and zanamivir for sialic acid release from *S. pneumoniae*

Because albuterol increased the antibiotic activity without markedly affecting the biomass but caused a variable degree of sialic acid release (both effects being reversed by zanamivir), we examined how albuterol could increase the activity of a purified neuraminidase A when acting on *S. pneumoniae*. Figure 7 shows that this was indeed the case for all 3 strains at concentrations of albuterol including those used for growing the biofilms and selected for the previous experiments. Zanamivir inhibited the neuraminidase activity and, in all cases, reversed the effect of albuterol.

DISCUSSION

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

Ipratropium and other choline analogues have been shown to inhibit pneumococcal growth and viability in planktonic cultures by interacting with choline-binding proteins like LytA amidase, LytC lyzozyme and Pce phosphocholinesterase [30]. LytA and LytC play a critical role in pneumococcal attachment to epithelia, tissue colonization and biofilm formation [26, 31]. 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 also suggest that this disassembly contributes to the increased activity of moxifloxacin, and, to a lesser extent, solithromycin, probably by improving the access of antibiotics to bacteria.

Moving now to albuterol, data suggest that this bronchodilator acts through matrix remodeling, mediated by the activation of neuraminidase A and, thereby, facilitating antibiotic diffusion. Indeed, zanamivir, a well known inhibitor of this activity, abolishes both the release of sialic acid induced by albuterol and its enhancing effect on antibiotic activity. Both the stimulatory effect of albuterol and the inhibitory effect of zanamivir could be reproduced *in vitro* with purified neuraminidase A. In *S. pneumoniae*, neuraminidase A is known to contribute to biofilm formation by cleaving sialic acid residues from glycans and mucin at the epithelial cell surface, exposing thereby host cell surface receptors for pneumococcal adherence [27, 32-34]. Sialic acid itself can also act as a signaling molecule, enhancing the bacterial adherence to surfaces and/or survival within biofilms [29]. Moreover, sialic acid is present in the intercellular matrix of pneumococcal biofilms [35]. As sialylated moieties are present on, or between, bacteria [36], the presence of free sialic acid in biofilm supernatants suggests a remodeling of the 3D-structure of the matrix and/or of interaction between bacteria during maturation.

The significance of our study is limited by the small number of strains examined, the use of a single molecule as representative of each bronchodilator and antibiotic classes, and the artificial nature of the support used for growing biofilms. Moreover, the effects of both ipratropium and albuterol on antibiotic activity were less marked for biofilms formed from the clinical isolate N6 compared to those formed from the two reference strains. This may result from differences in matrix composition or tridimensional structure, which are described as strain and serotype-dependent [5, 35, 37]. Moreover, albuterol effects may also depend on the baALB neuraminidase A expression or activity, since the expression of this enzyme varies among serotypes as described for other streptococcal species [38]. We also did not test for the effect of corticosteroids because we observed that budesonide, a typical inhaled corticosteroid, exerts it-self an antibacterial effect on planktonic cells (with MICs of about 3-6 mg/L), confirming literature data [39]. As they are, however, our

results already clearly demonstrate the beneficial effect of ipratropium, and to some extent, albuterol and their synergy with moxifloxacin and solithromycin. Of interest is the observation that the two bronchodilators are not antagonists in this context. We have no simple explanation concerning the lower and almost complete absence of effects seen when biofilms are exposed to amoxicillin or clarithromycin as opposed to solithromycin and, most strikingly, moxifloxacin. For moxifloxacin, this may be related to the well-known fluoroquinolones intense bactericidal activity. It is tempting to speculate that the effects described here for moxifloxacin may at least partly explain why this antibiotic is superior to either amoxicillin or clarithromycin for clinical cure, bacteriologic eradication, and long-term outcomes of AECB in patients suffering from COPD [17]. Similar clinical studies with solithromycin, which binds more tightly to the 50S ribosome subunit than clarithromycin [40] would, therefore, be of large interest. More broadly speaking, the present data may help further supporting and rationalizing the current GOLD guidelines that recommend associating antibiotics with short-acting bronchodilators for the treatment of bacterial exacerbations [14] in grade A patients (as first choice) or for all grades as alternative treatments.

Ethical approval

The entire study protocol was submitted and approved by the *Commission d'éthique facultaire* of the *Université catholique de Louvain* (unique Belgian no. 40320109783) and the ethical committee of each participating hospital.

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Potential conflicts of interest:

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Table 1: 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 2: 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] albuterol, fenoterol, formoterol, ALBmeterol, indacaterol

[b] ipratropium, thiotropium

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

[d] formoterol, ALBmeterol, indacaterol, thiotropium

[e] budesonide, fluticasone, beclomethasone

Figure 1

Caption to Figure 1: Kinetics of biofilm formation (biomass, as evaluated by crystal violet absorbance OD_{570nm}) by the reference capsulated strain ATCC49619 (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 cultivated in control conditions (open circles), or in medium supplemented with 1.45 mg/L ipratropium (downside closed triangles), 7.25 mg/L albuterol (upside closed triangles), or the combination of ipratropium (1.45mg/L) and albuterol (7.25 mg/L) (grey squares). All values are means ± standard error of the mean (SEM) of 3 to 26 experiments (each made 12 times; when not visible, the SEM bars are smaller than the size of the symbols). Data were used to fit a sigmoidal dose-response function whenever possible (dotted straight lines are used when changes in OD_{570nm} occurred abruptly). An inverted sigmoidal function (slope factor =1) was used for describing the decrease of OD_{570nm} observed between days 6 and 11 for the biofilm grown from strain N6 in the presence the combination of ipratropium and albuterol.

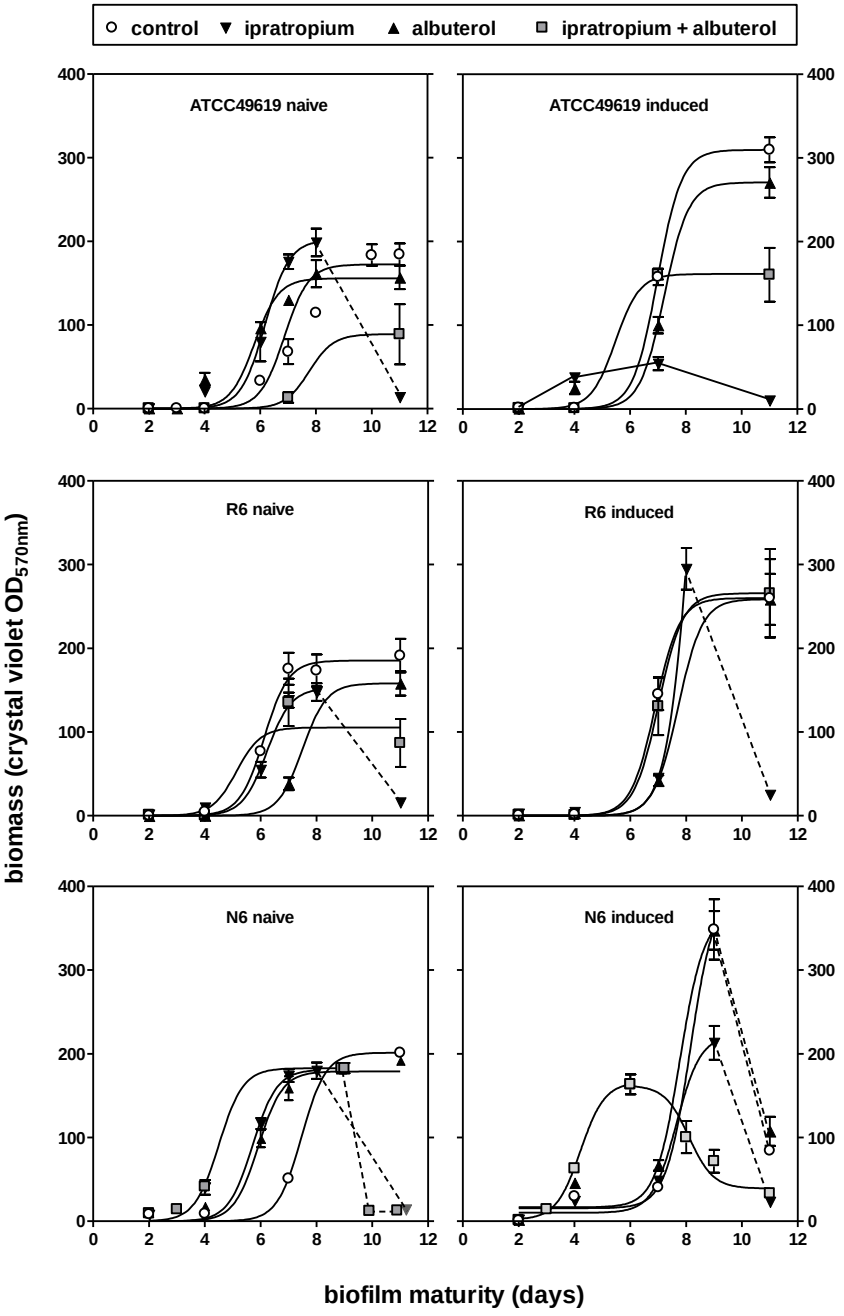


Figure 2

Caption to Figure 2: Concentration-response activity of amoxicillin, clarithromycin, solithromycin and moxifloxacin on viability (left) and biomass (right) against 2-days naive and 11-days induced biofilms produced from strain ATCC49619 grown in control conditions or in the presence of a combination of 7.25 mg/L albuterol and 1.45 mg/L ipratropium. The ordinate shows the change in viability (resorufin fluorescence) or in biomass (crystal violet OD_{570nm}) in percentage of the values observed in the absence of antibiotic. The abscissa shows the concentration range investigated in multiples of the minimal inhibitory concentrations (MIC) of the corresponding antibiotics in broth (0.064, 0.032, 0.008 and 0.125mg/L for amoxicillin, clarithromycin, solithromycin and moxifloxacin, respectively). All values are means ± standard deviation (SD) of 3 to 8 replicates (when not visible, the SD bars are smaller than the size of the symbols). Data were used to fit a sigmoidal dose-response curves (slope factor = 1).

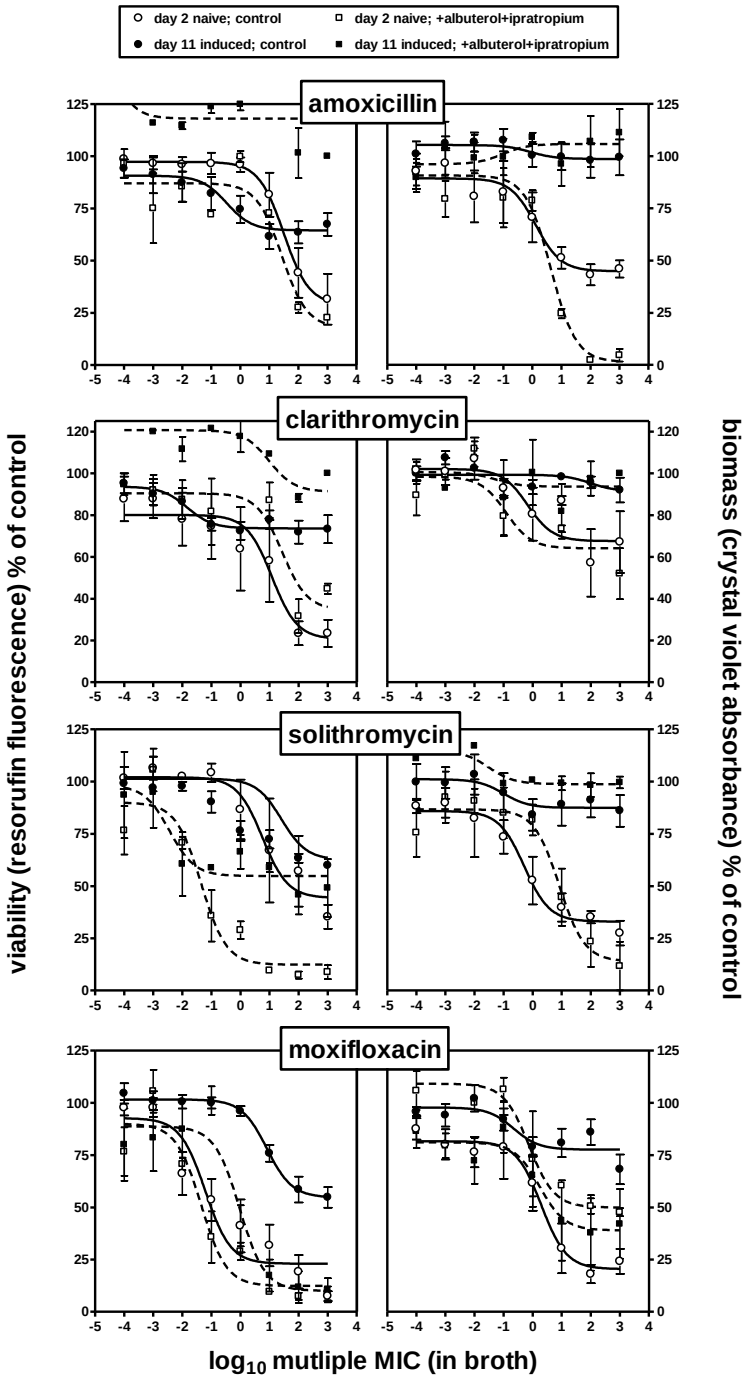


Figure 3

Caption to Figure 3: Concentration-response activity of moxifloxacin (3A) and solithromycin (3B) on viability (left) and biomass (right) against 11-days old induced biofilms produced from strain ATCC49619, R6 and N6 grown in control conditions or in the presence of 7.25 mg/L albuterol or 1.45 mg/L ipratropium. The ordinate shows the change in viability (resorufin fluorescence) or in biomass (crystal violet OD_{570nm}) in percentage of the values observed in the absence of antibiotic. The abscissa shows the concentration range investigated in multiple of the minimal inhibitory concentrations (MIC) of the corresponding antibiotics in broth (0.125 and 0.008 mg/L [ATCC49619], 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 ± standard error of the mean (SEM) of 2 to 8 determinations performed each in quadruplicates (when not visible, the SEM bars are smaller than the size of the symbols). Data were used to fit a sigmoidal dose-response curves (slope factor = 1), with the pertinent pharmacological descriptors presented in online data supplement (see Supplementary Tables 1-3).

Figure 3A

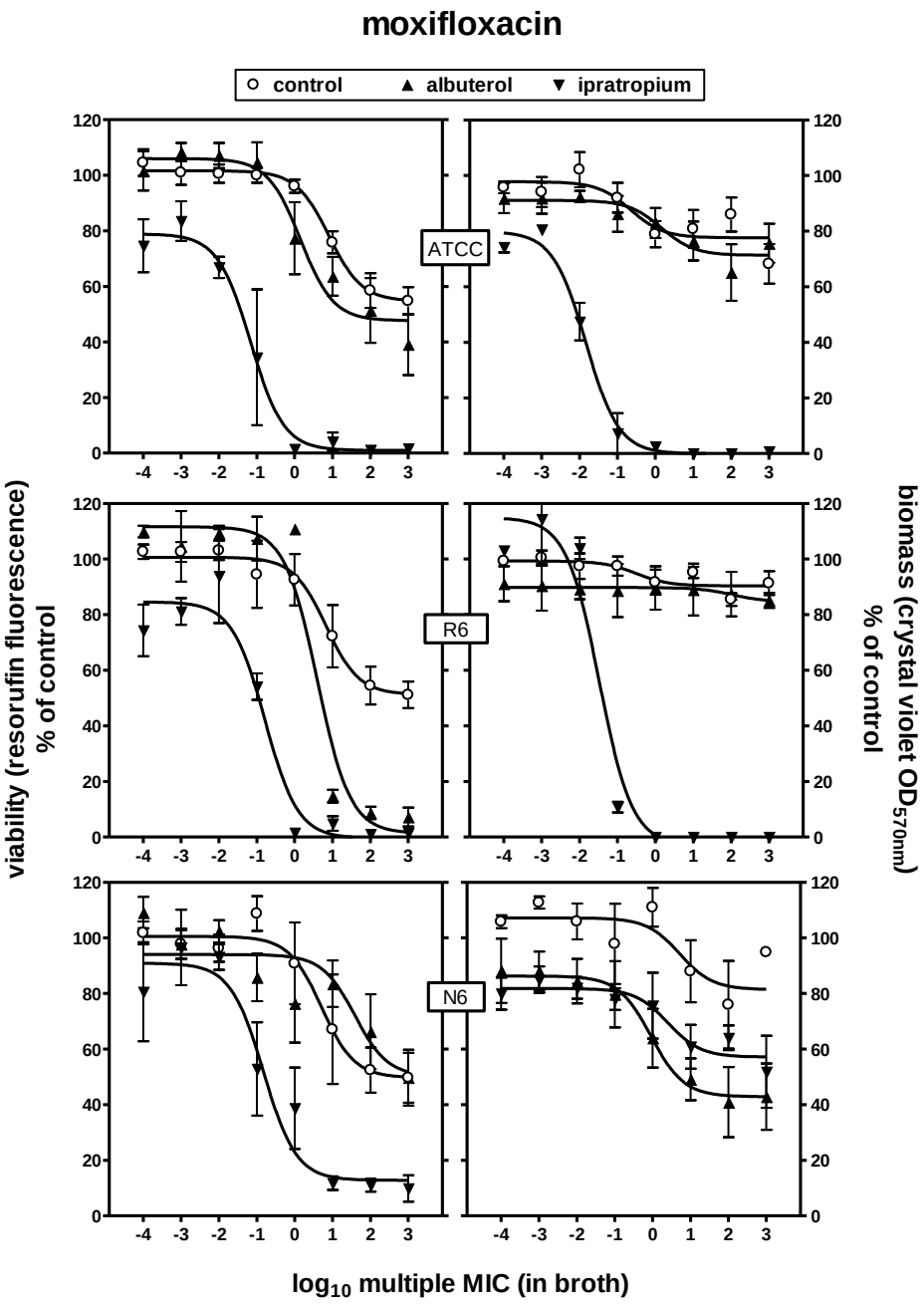


Figure 3B

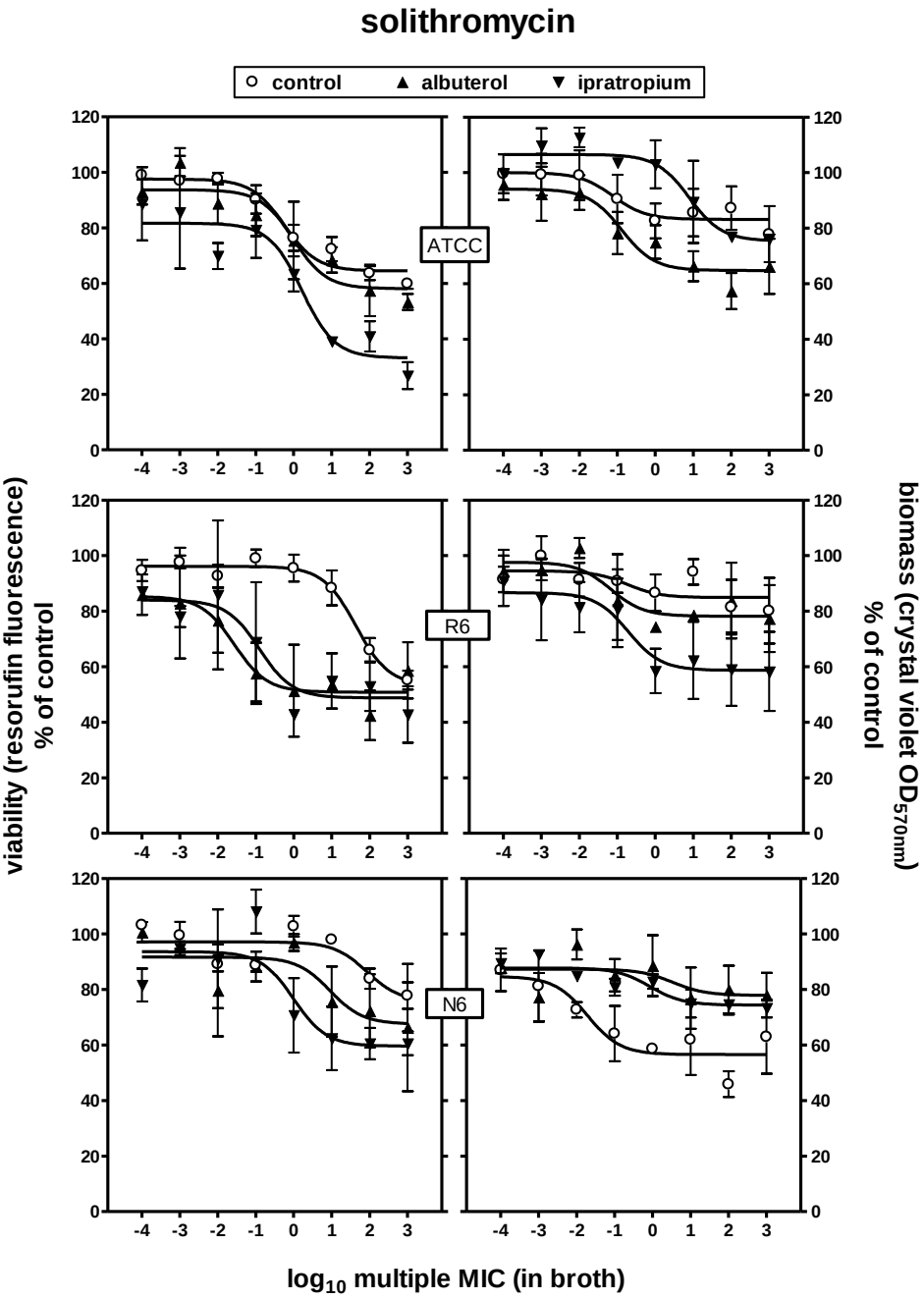

solithromycin
control
albuterol
ipratropium
ATCC
R6
N6
viability (resorufin fluorescence)
% of control
biomass (crystal violet OD570nm)
% of control
log10 multiple MIC (in broth)

Figure 4

Caption to Figure 4: Concentration-response activity of moxifloxacin (left panels) and solithromycin (right panels) on viability against 2-days old induced biofilms produced from strain ATCC49619, R6 or N6 grown in control conditions or in the presence of 7.25 mg/L albuterol or 7.25 mg/L of albuterol + 250 mg/L zanamivir. The ordinate shows the change in viability (resorufin fluorescence) in percentage of the values observed in the absence of antibiotic. The abscissa shows the concentration range investigated in multiple of the minimal inhibitory concentrations (MIC) of the corresponding antibiotics in broth (0.125 and 0.008 mg/L [ATCC49619], 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 ± standard error of the mean (SEM) of 2 to 8 determinations performed each in quadruplicates (when not visible, the SEM bars are smaller than the size of the symbols). Data were used to fit a sigmoidal dose-response curves (slope factor = 1), with the pertinent pharmacological descriptors presented in online data supplement (see Supplementary Tables 1-3).

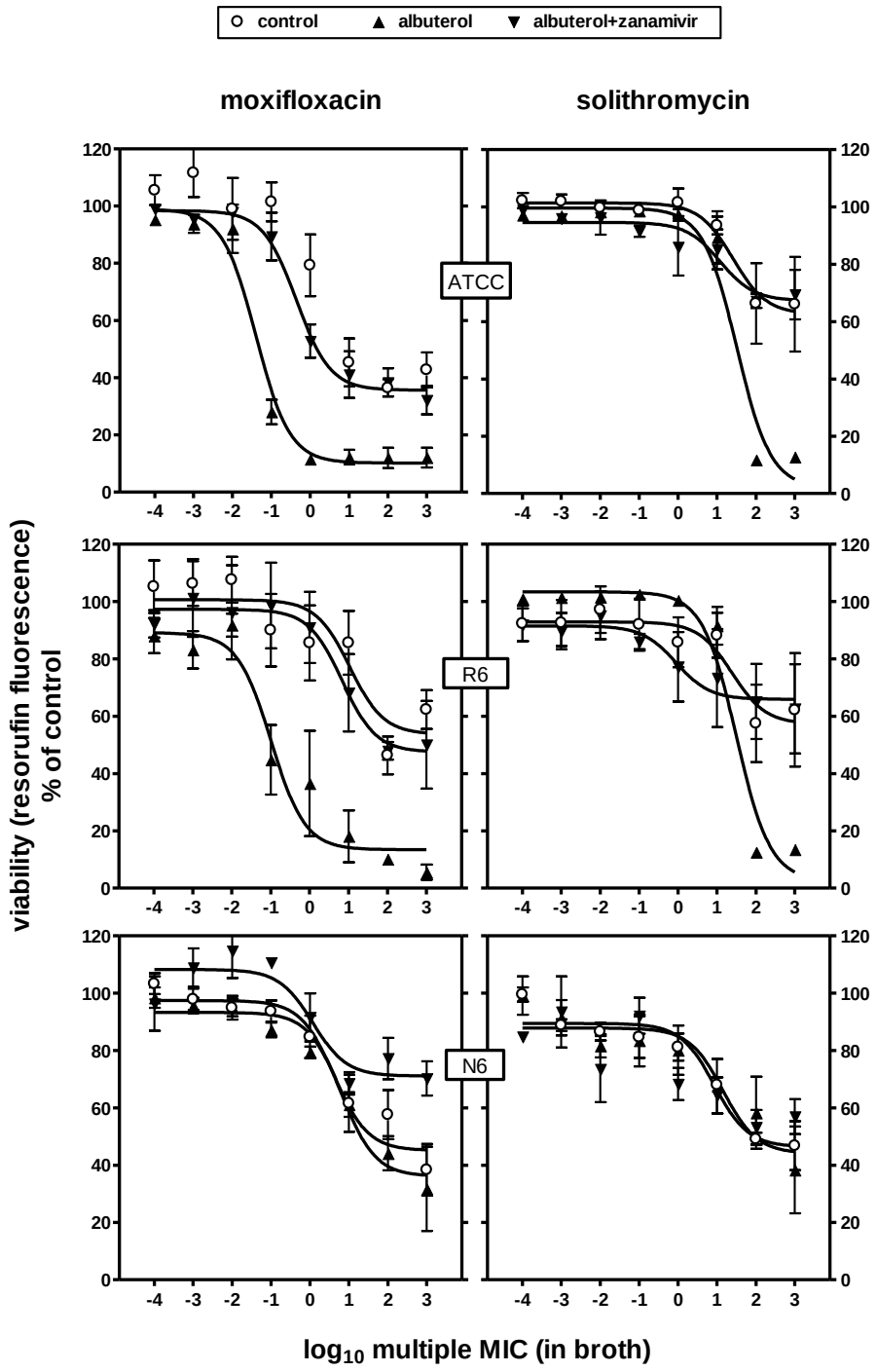


Figure 5

Caption to Figure 5: Free sialic acid concentrations in the supernatant of 11-days old naive or induced biofilms grown in control medium or in medium supplemented with ipratropium (1.45 mg/L) as a function of the biomass (left panel) and of bacterial residual viability upon incubation with moxifloxacin (middle panel) or solithromycin (right panel). as % of control (using E_{max} values ; see Figures 3A and 3B, and Table SP1]).

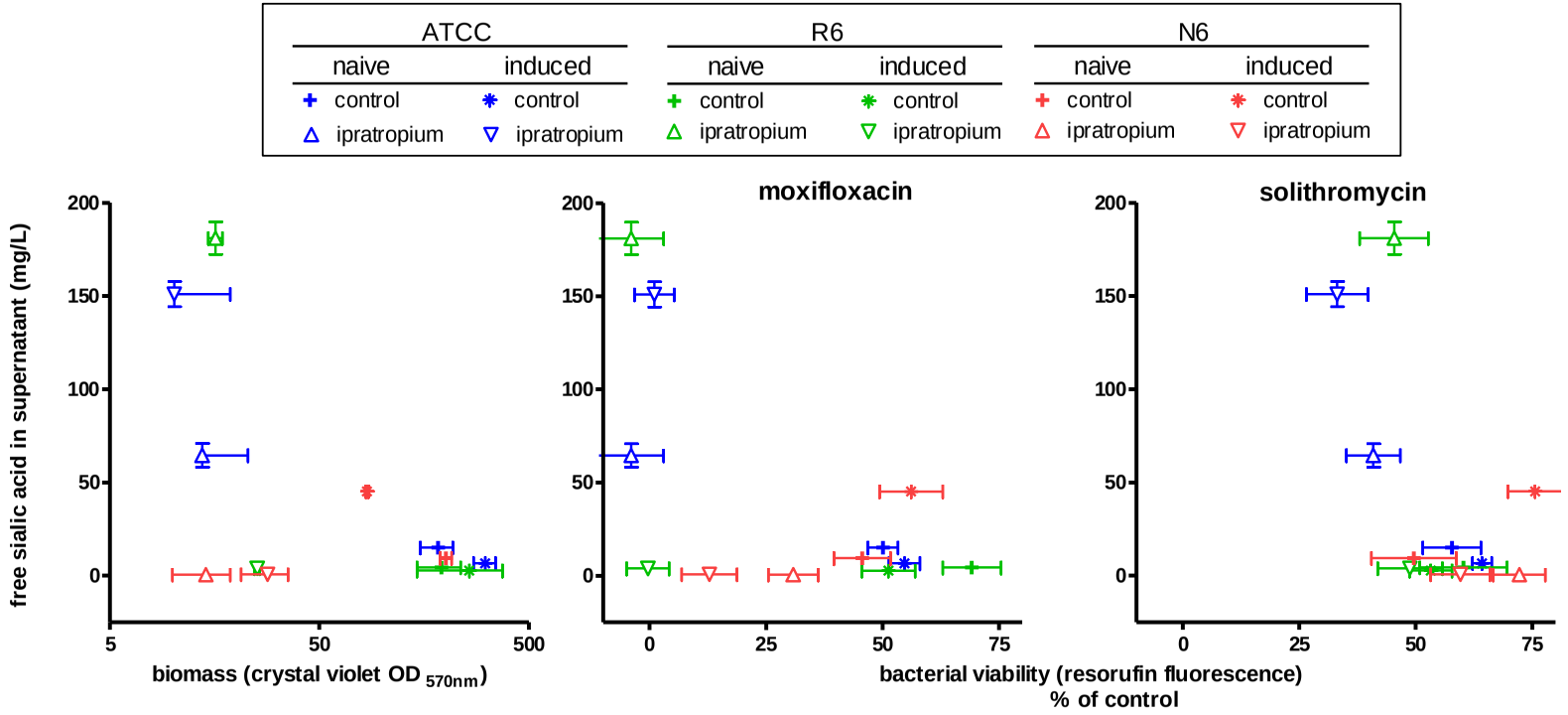
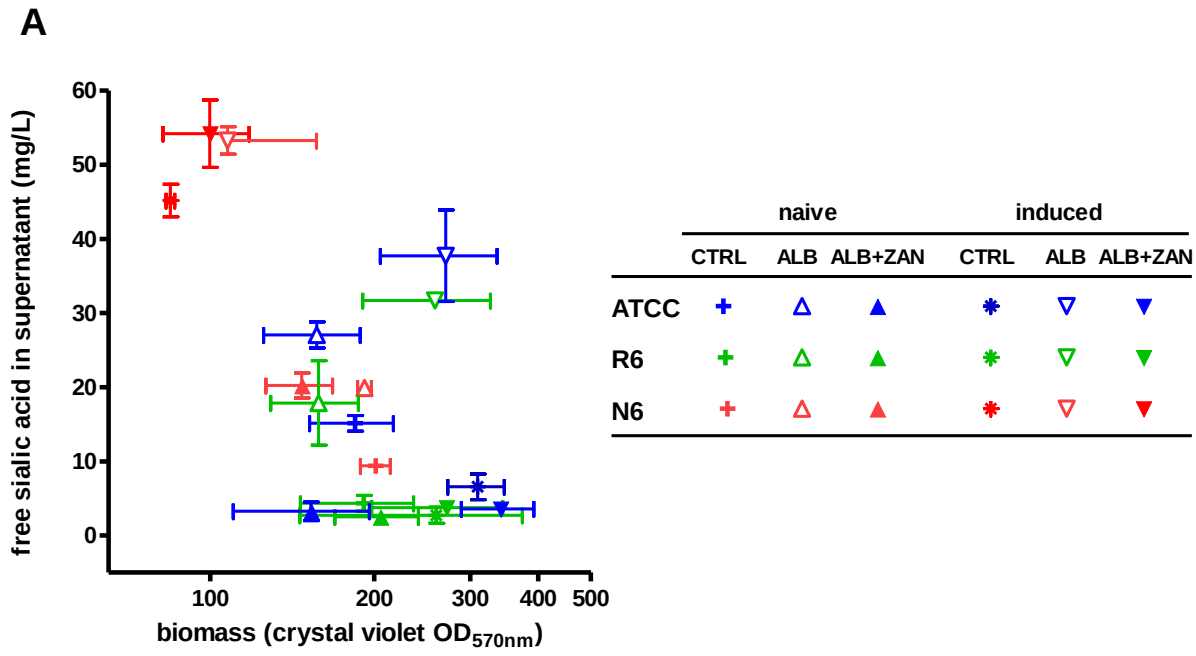
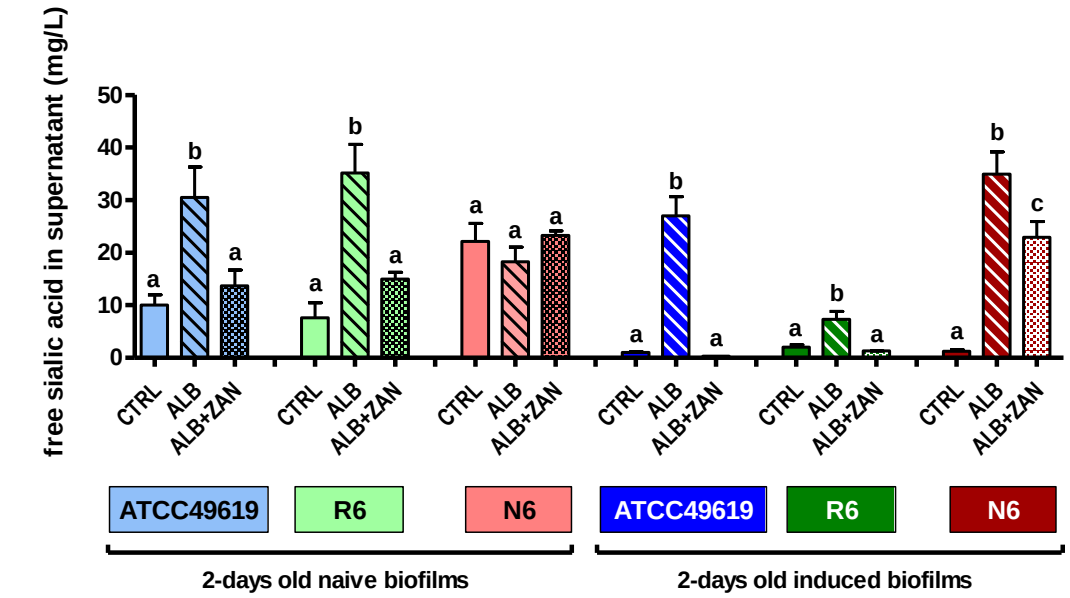


Figure 6 (two panels)

Caption to Figure 6: A: correlation between the concentration of free sialic acid in the supernatant of 11-days old naive or induced biofilms grown in control medium (CTRL) or in medium supplemented with albuterol (ALB; 7.25 mg/L) or with albuterol (7.25 mg/L) and zanamivir (250 mg/L) (ALB+ZAN) and biomass amounts. B: Influence of albuterol (ALB; 7.25 mg/L) or of albuterol (7.25 mg/L) combined with zanamivir (250 mg/L) (ALB+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-days (upper histogram) and 11-days (lower histogram) naive and induced biofilms. Statistical analysis (one-way ANOVA with Tukey's post-test): in each group, bars with different letters indicate significant differences between media ($p < 0.05$).



B


free sialic acid in supernatant (mg/L)
50
40
30
20
10
0
a
b
a
a
b
a
a
a
a
a
b
a
a
b
a
a
b
c
CTRL
ALB
ALB+ZAN
ATCC49619
R6
N6
2-days old naive biofilms
2-days old induced biofilms

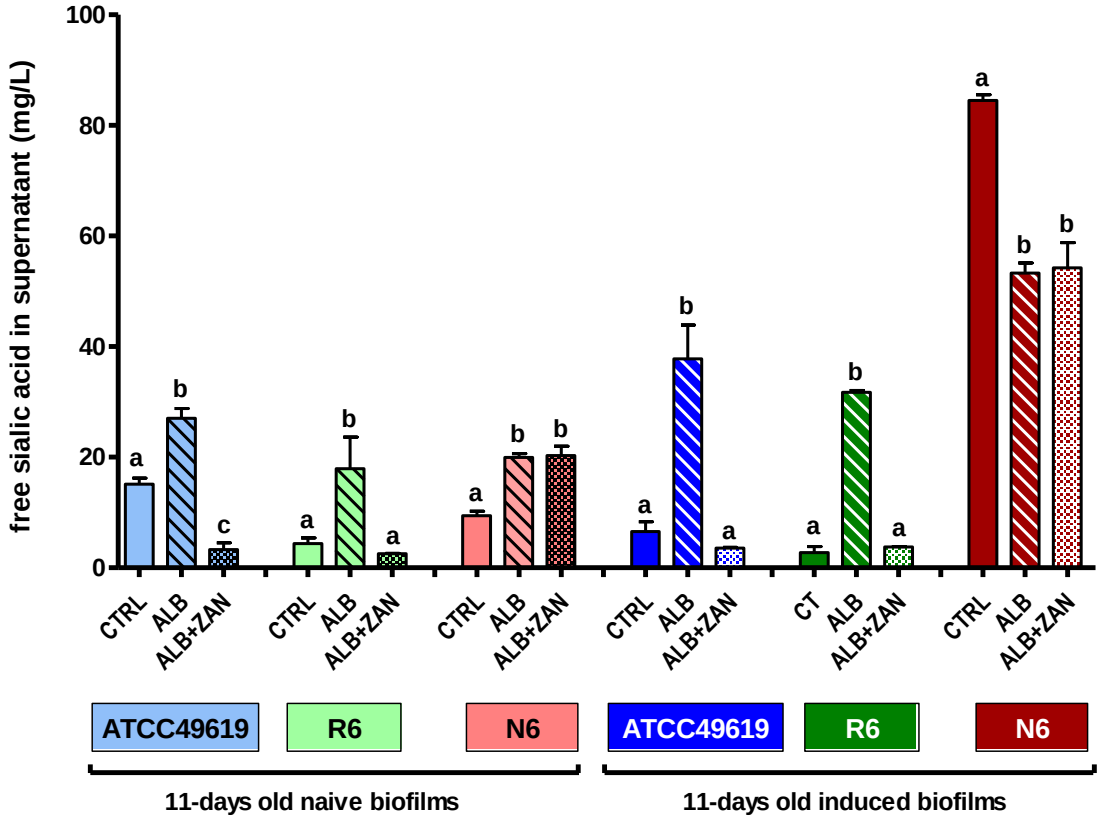
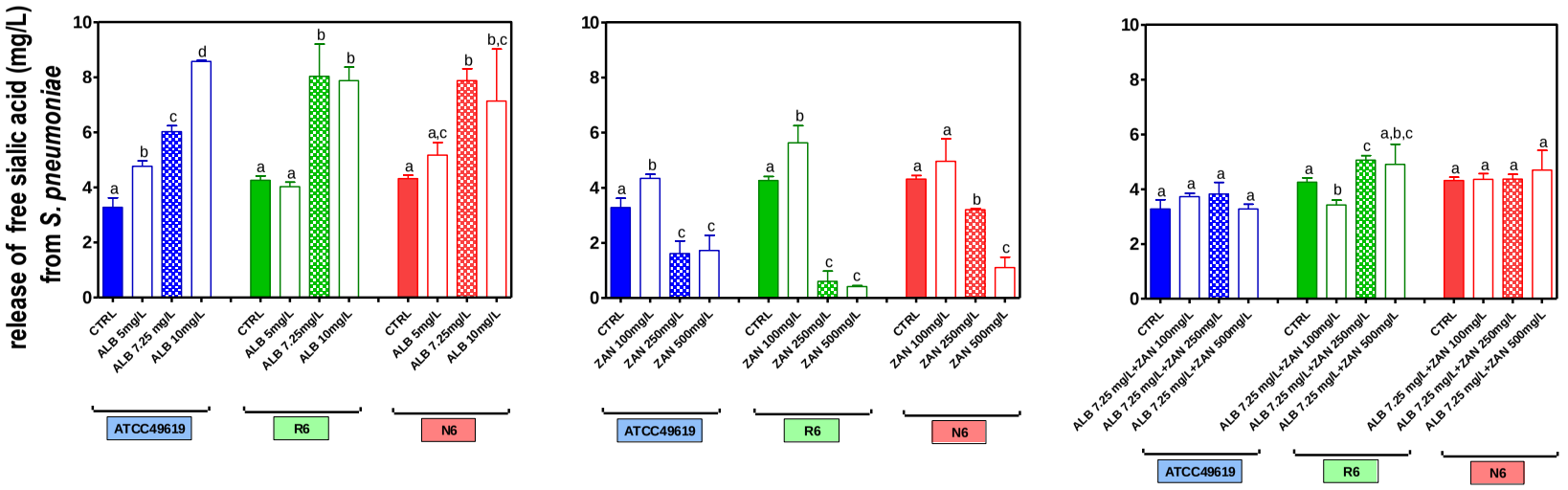

free sialic acid in supernatant (mg/L)
100
80
60
40
20
0
a
b
c
a
b
a
a
b
b
a
b
a
a
b
a
a
b
b
a
b
b
CTRL
ALB
ALB+ZAN
CT
ATCC49619
R6
N6
11-days old naive biofilms
11-days old induced biofilms

Figure 7

Caption to Figure 7: Amounts of sialic acid (mg/L) released from *S. pneumoniae* collected from the supernatant of 2-days old naive biofilms (made by reference strains ATCC49619, R6 and clinical AECB isolate N6) by purified *Arthrobacter ureafaciens* α-(2→3,6,8,9)-neuraminidase alone or in the presence of albuterol, zanamivir, or their combination. CTRL (full bars): control conditions (no addition); ALB, ZAN, ALB+ZAN: addition of albuterol, zanamivir, or their combination at the concentrations indicated in the abscissa. Statistical analysis (one-way ANOVA with Tukey's post-test): in each group, bars with different letters indicate significant differences between conditions ($p < 0.05$). Albuterol and/or zanamivir concentrations used for biofilm culture during studies of biofilm development and antibiotic activity are represented with stippled bars, other concentrations used in this experiment are represented with open bars.



Supplementary Material

Supplementary Text #1: Details of choice for albuterol, ipratropium and zanamivir doses used in this study

In this study, we used different culture media for biofilm growth, supplemented or not with albuterol (7.25mg/L) and/or ipratropium (1.45 mg/L). These concentrations were chosen to mimic those expected to be measured in the epithelium lining fluid upon single administration (albuterol [2.5mg]; ipratropium [0.5mg]) by considering (i) a mean pulmonary deposition of 10% (see Product Characteristics of Combivent® [association albuterol+ipratropium] (1)), and (ii) a mean epithelial lining fluid volume of 34.5mL (2). Zanamivir, an inhibitor of pneumococcal neuraminidase was used at 250 mg/L, a concentration demonstrated during pilot studies as inhibiting biofilm formation by >50%; higher concentrations did not improve this effect. Similar observations are reported in the literature (3).

Supplementary Text #2: Assay of free sialic acid in biofilm supernatant – Specifications for supernatant purification and HPLC-MS assay

Biofilm supernatant was centrifuged at 14,000RPM for 10 min (Eppendorf centrifuge 5417R, rotor DL 039, Eppendorf AG, Hamburg, Germany) and the supernatant mixed with an equal volume of acetone (98.5% purity; Merck AG, Darmstadt, Germany]). After centrifugation, the supernatant was collected, flushed with a gentle flow of air at room temperature until removal of acetone. The residual aqueous phase was again mixed with an equal volume of acetone and subjected to the same process three times, and the final aqueous phase used for determination of its content in sialic by HPLC-MS and enzymatic assays (see full text).

The HPLC-MS assay was performed using an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, Waltham, MA) coupled to an Accela HPLC system (ThermoFisher Scientific, Waltham, MA). HPLC specifications were as follows: 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 a gradient from 10% B to 70% B linearly over 15 min, 15 min at 70% B, re-equilibration at 10% B. MS analysis was performed in the negative mode with an ESI ionization 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 the accuracy of the first method and then added to selected samples before purification.

References:

(1) Boehringer Ingelheim (Canada) Ltd. COMBIVENT UDV Product Monograph. **2014**.

(2) Rennard SI, Basset G, Lecossier D, et al. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. J Appl Physiol (1985) **1986**; 60(2):532-8.

(3) Trappetti C, Kadioglu A, Carter M, et al. Sialic acid: a preventable signal for pneumococcal biofilm formation, colonization, and invasion of the host. J Infect Dis **2009**; 199(10):1497-505.

Supplementary Table 1. 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 with CI at 95%)	Concentration (X MIC - mg/L) yielding 50% reduction	R^2	E_{max}[b] (% loss of matrix with 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
ALB	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
ALB+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
ALB+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 ($p<0.05$).

Supplementary Table 2. 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 with CI at 95%)	Concentration (X MIC - mg/L) yielding 50% reduction	R^2	E_{max}[b] (% loss of matrix with 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
ALB	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
ALB+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
ALB+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 ($p<0.05$).

Supplementary Table 3. 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 with CI at 95%)	Concentration (X MIC - mg/L) yielding 50% reduction	R^2	E_{max}[b] (% loss of matrix with 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
ALB	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
ALB+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
ALB+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

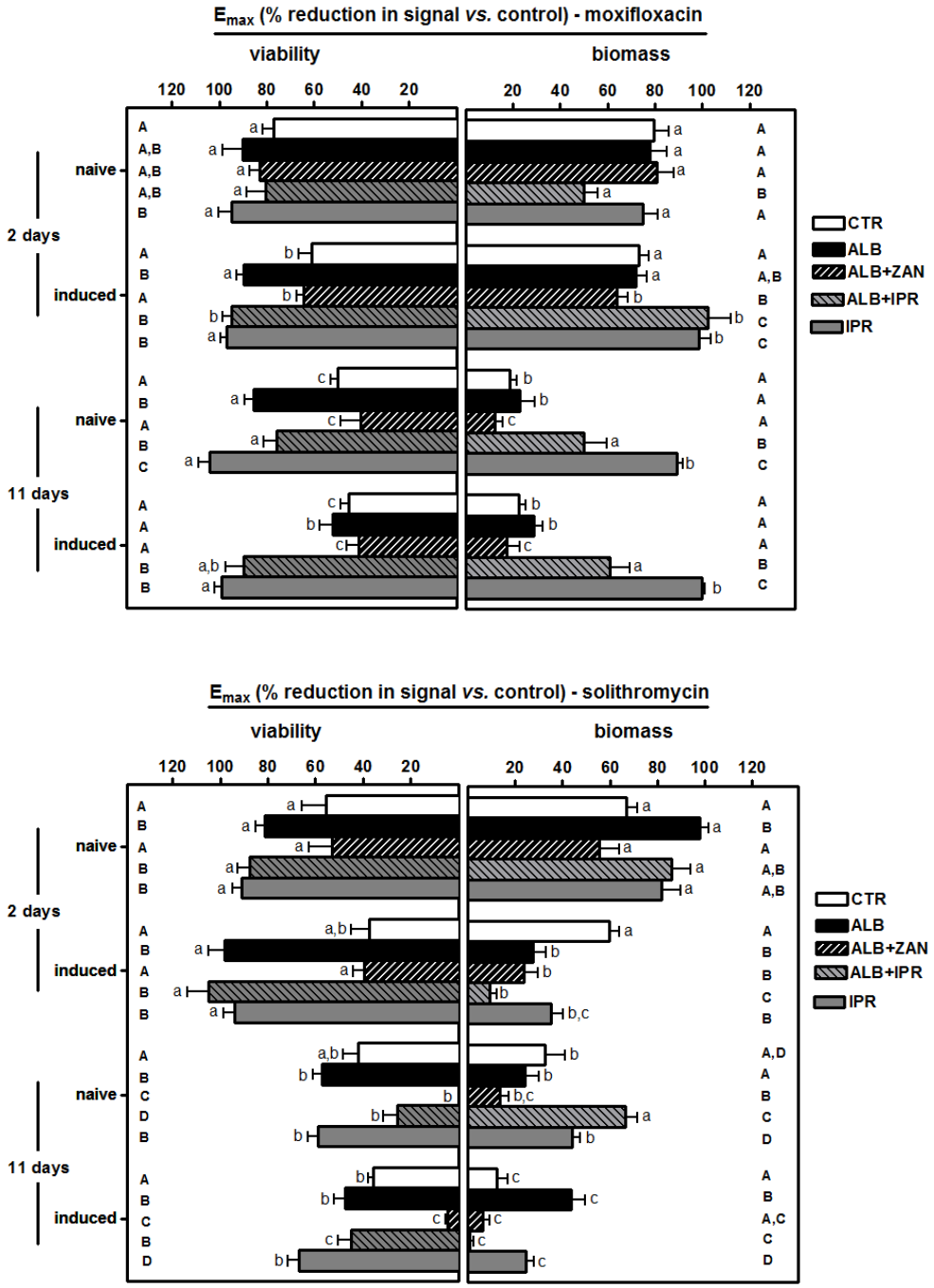
[a] Calculated based on sigmoidal regressions with a Hill coefficient of 1

[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 ($p<0.05$).

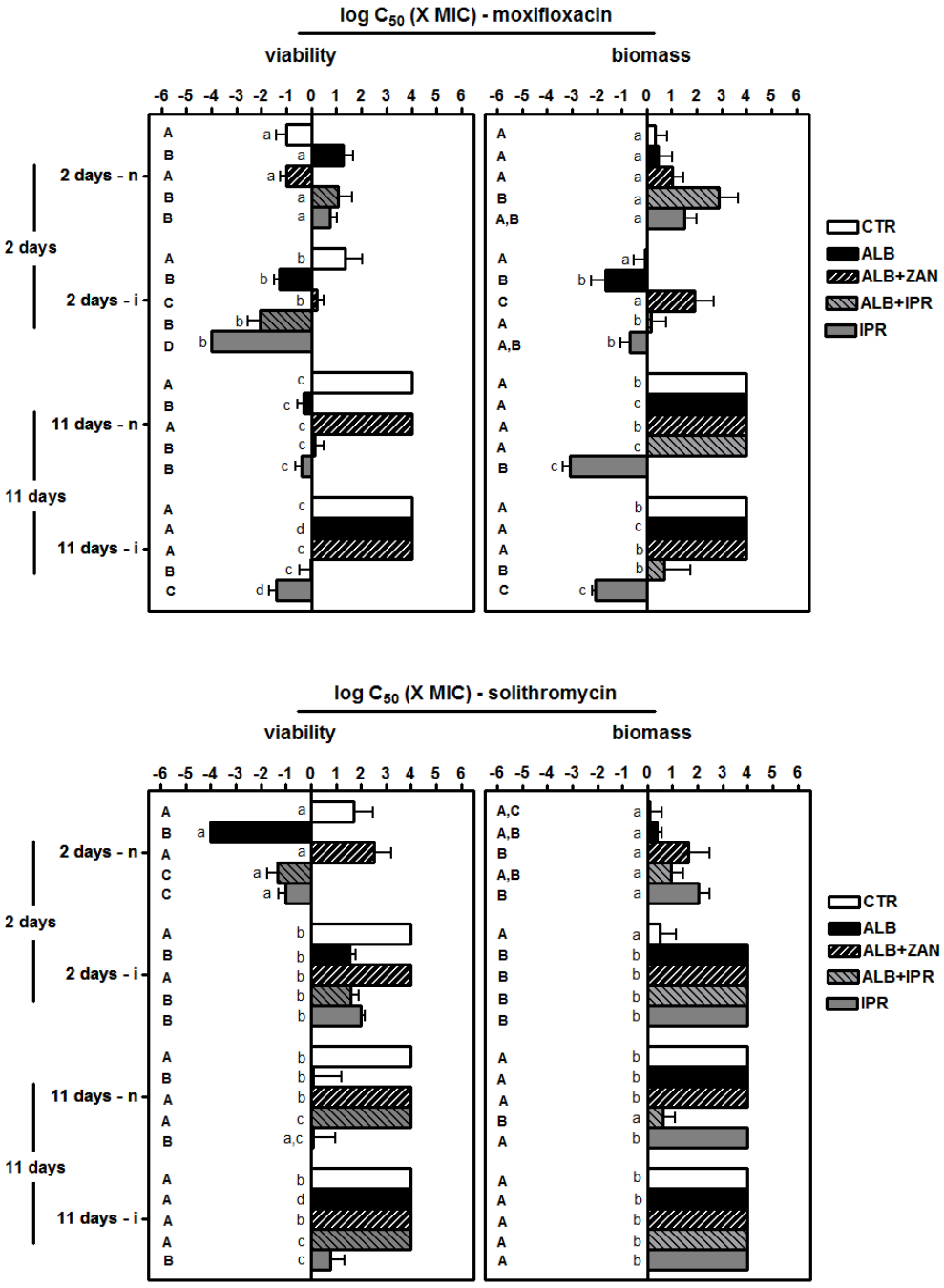
Supplementary Figure 1

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 Supplementary Table 1 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.



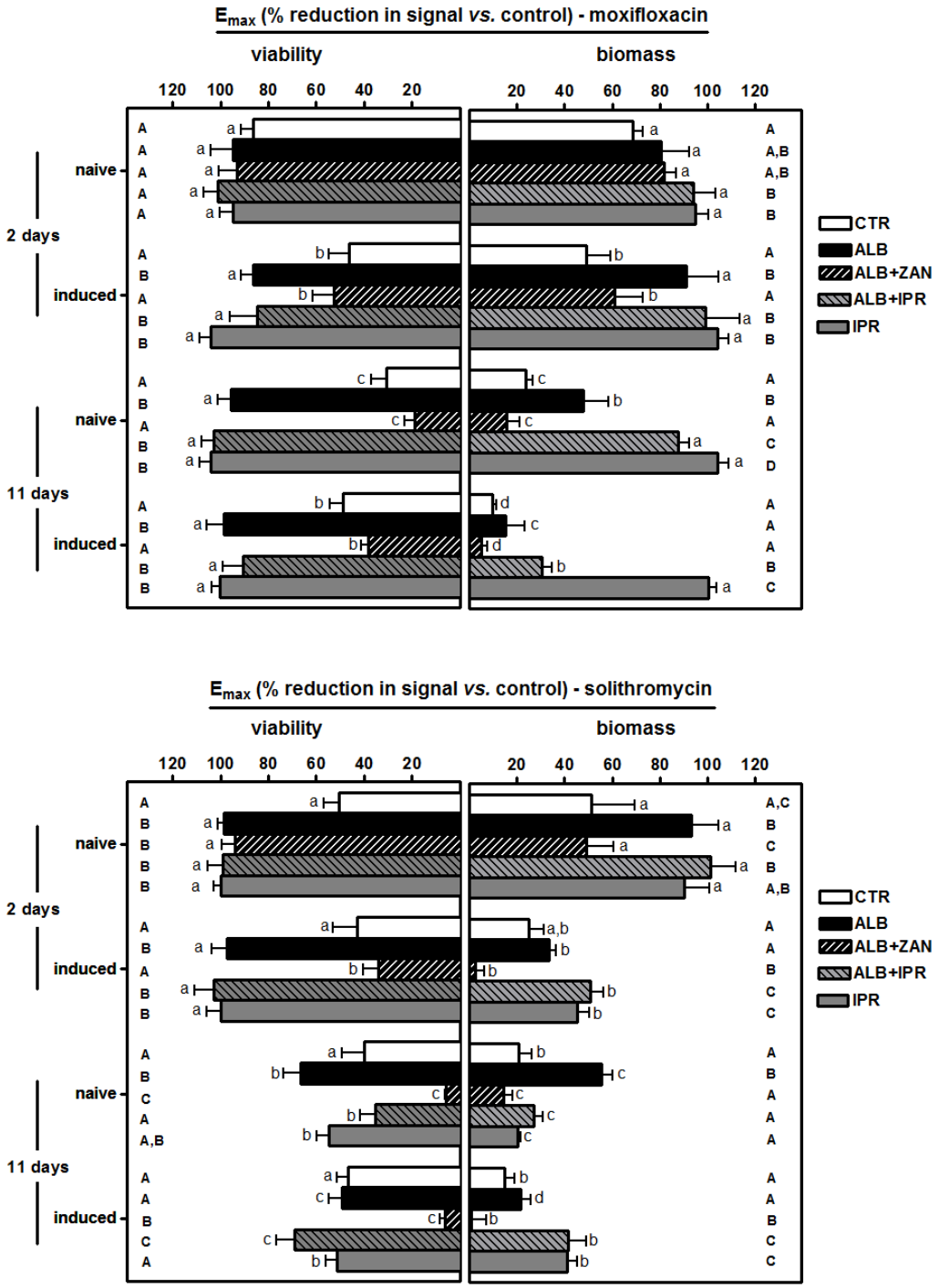
Supplementary Figure 2

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 E1 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.



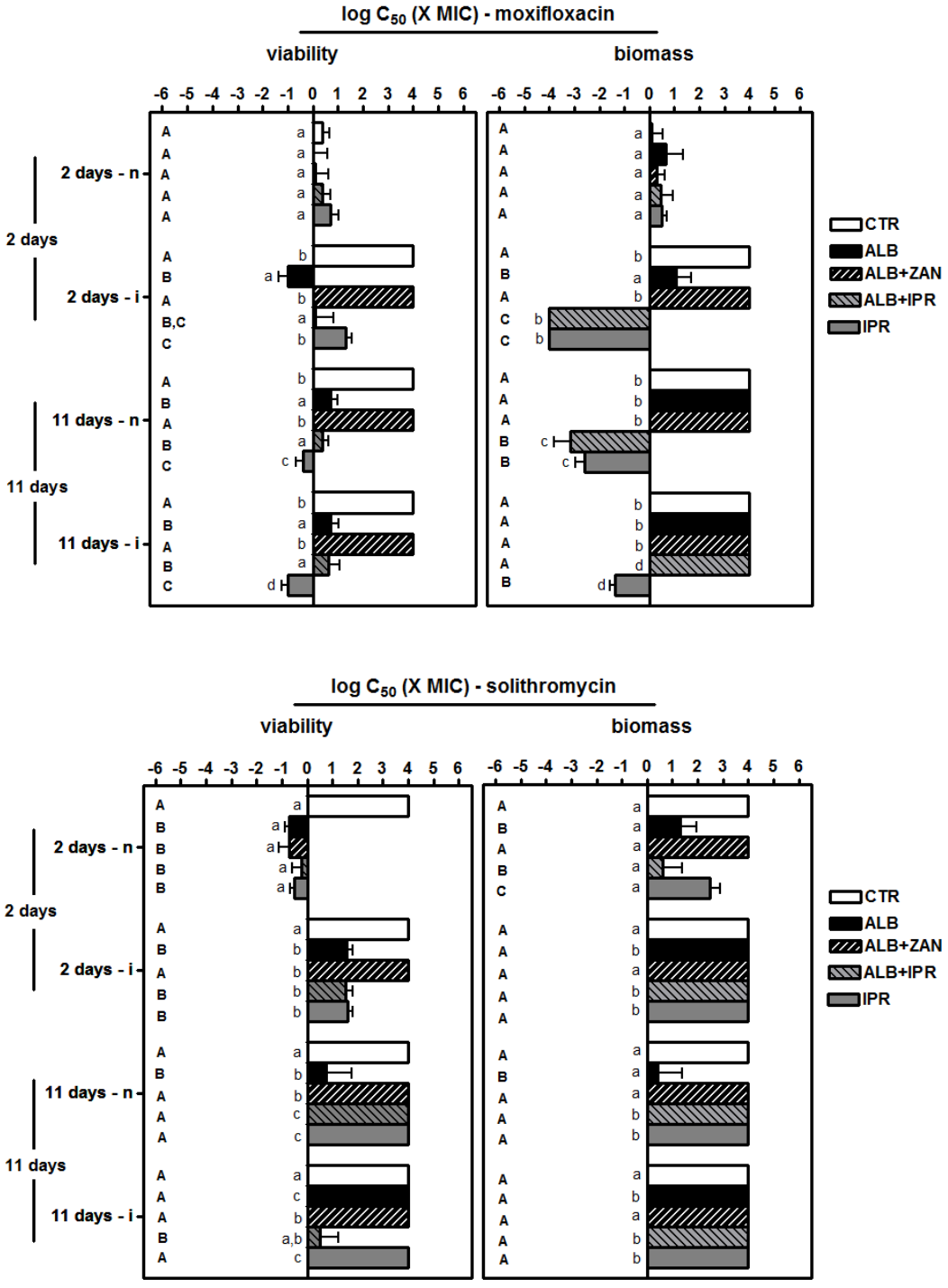
Supplementary Figure 3

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 Supplementary Table 2 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.



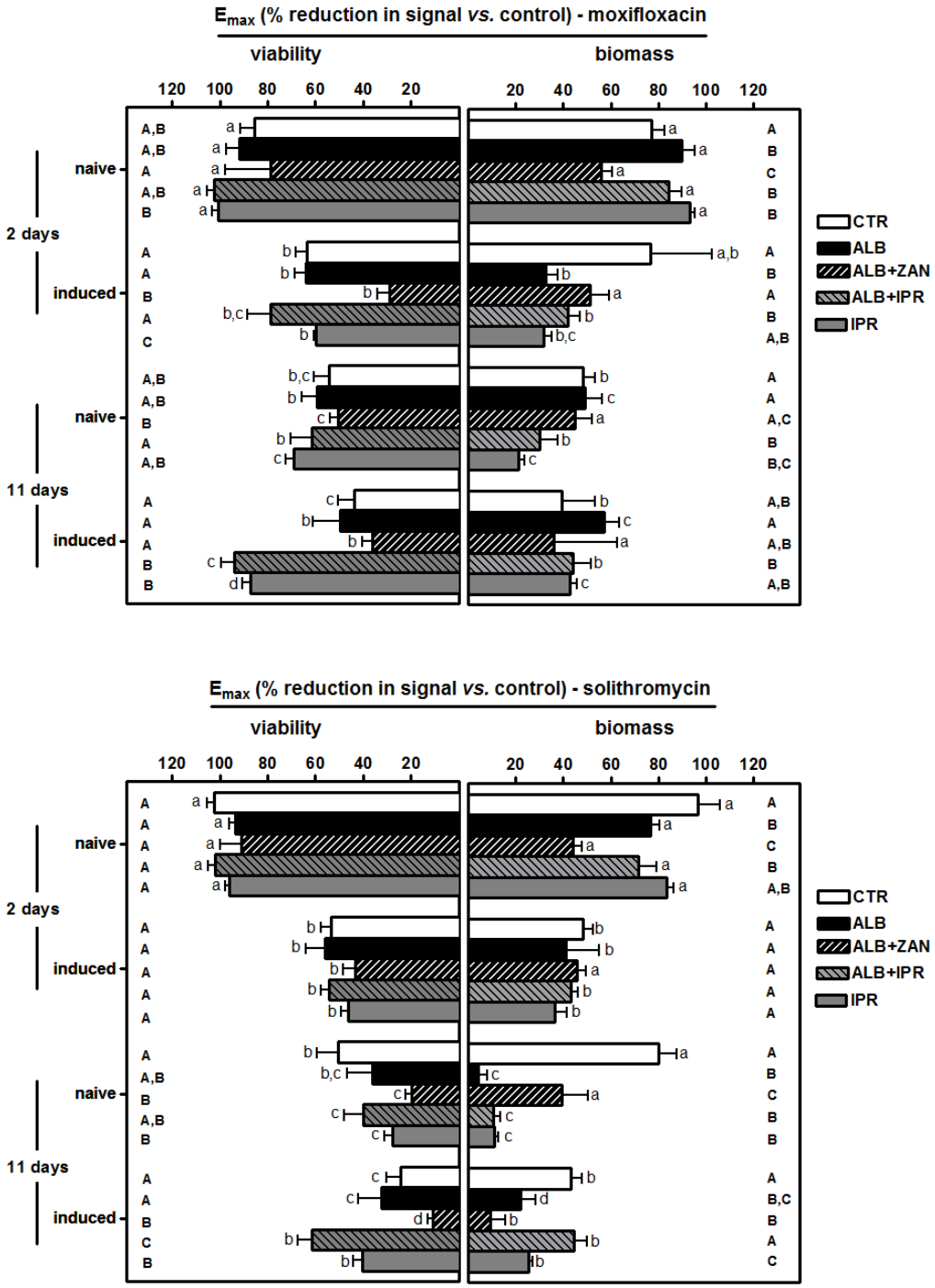
Supplementary Figure 4

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 Supplementary Table 2 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.



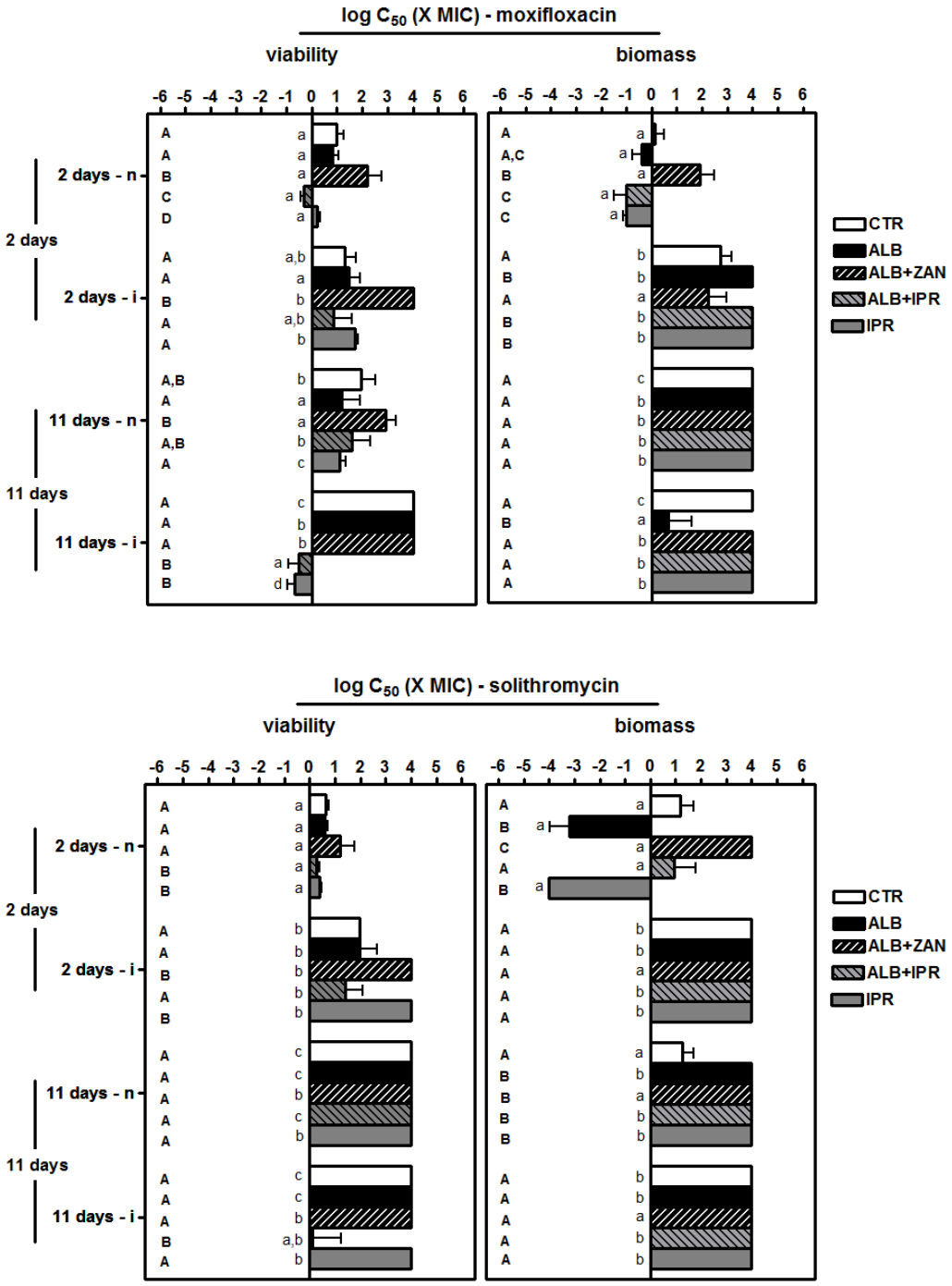
Supplementary Figure 5

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 Supplementary Table 3 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.



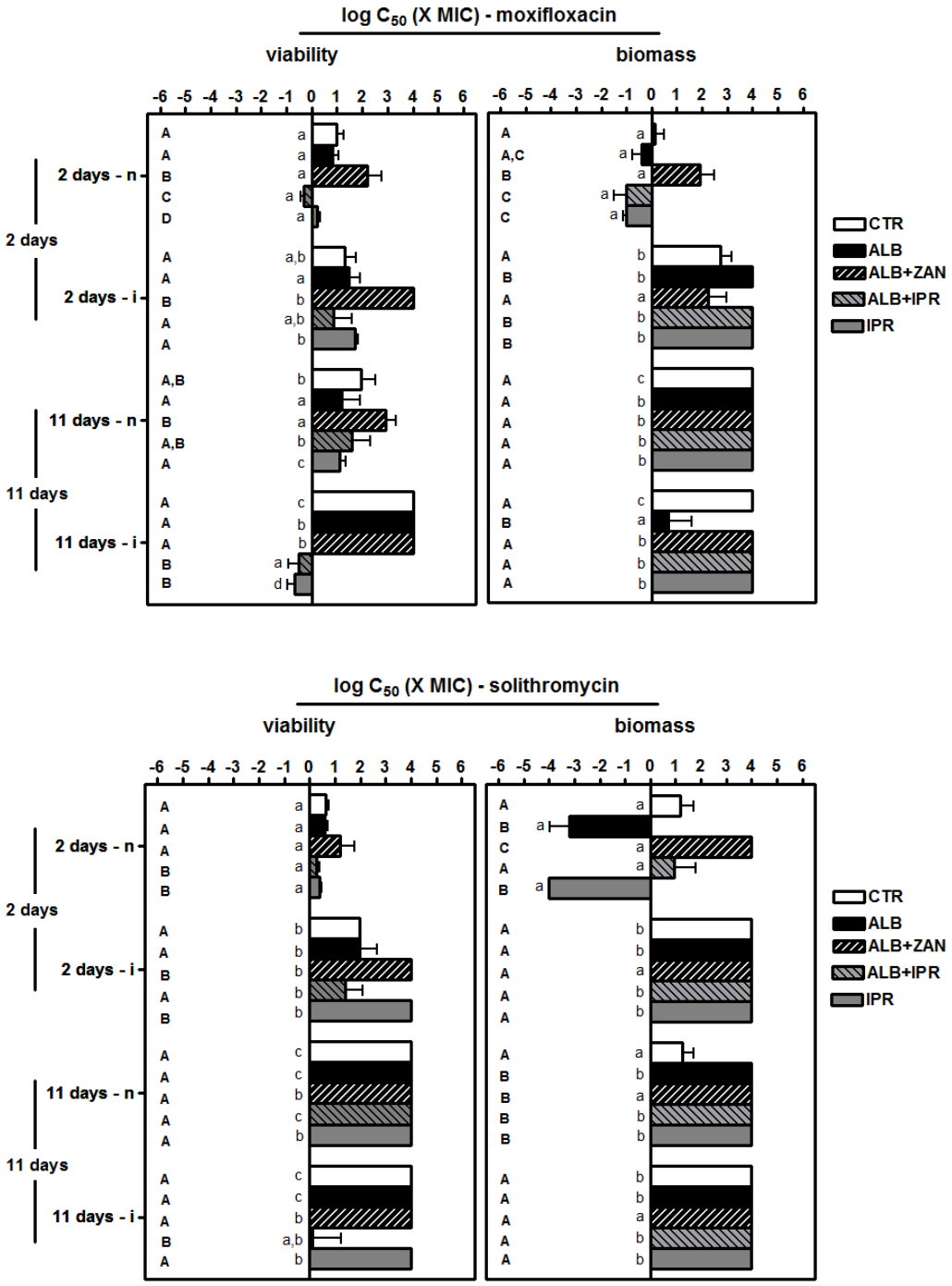
Supplementary Figure 6

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 Supplementary Table 3 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.



Supplementary Figure 6

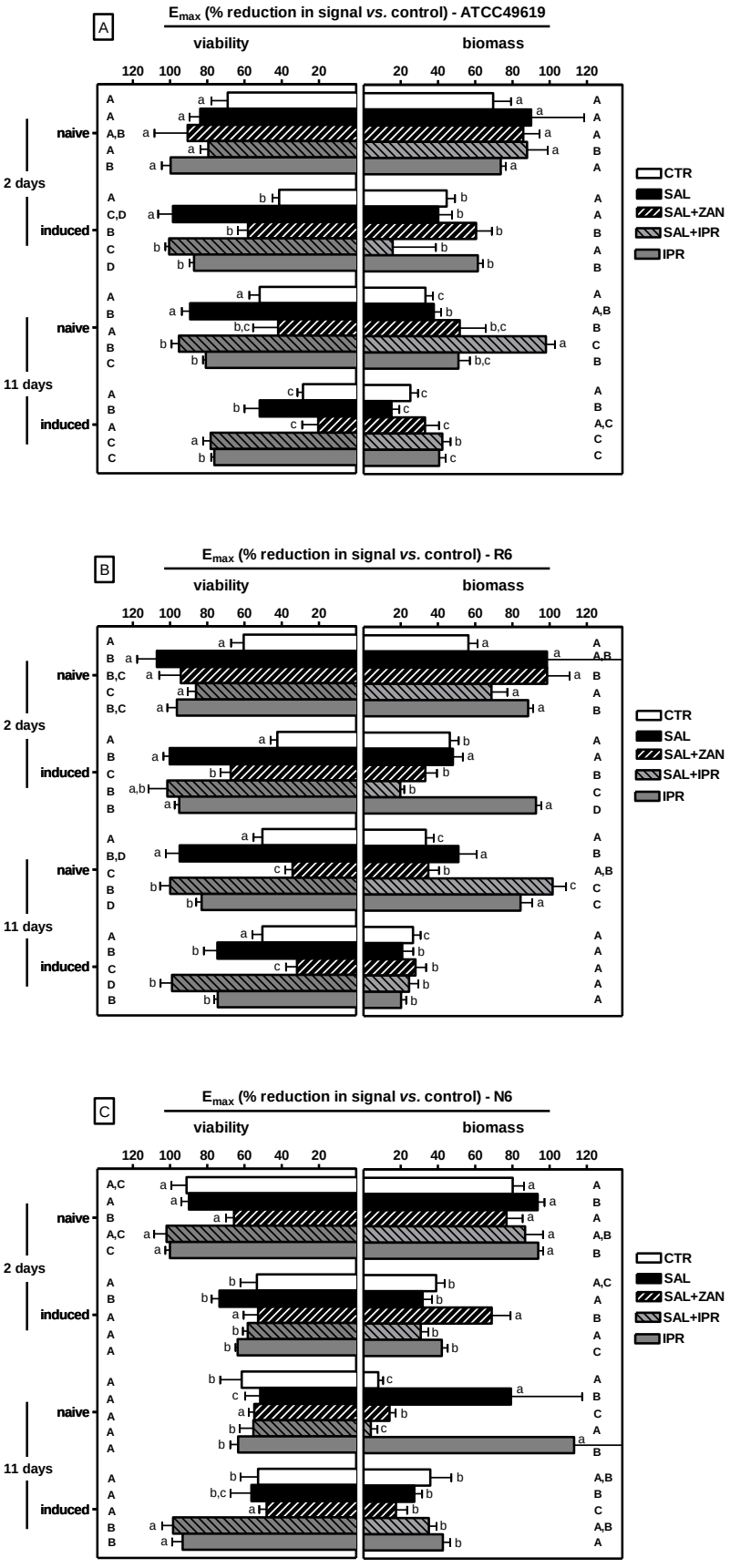
Comparison of antibiotic maximal relative potencies (C_{50}) expressed in X MIC towards viability (left panels) or biomass (right panels) for 2-days and 11-days old naive (n) and induced (i) biofilms of strain N6. Top panels: moxifloxacin; bottom panels, solithromycin. Biofilms were grown Values were 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 Supplementary Table 3 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.



The impact of ipratropium and salbutamol on biofilm matrix cohesion allowing improvements of moxifloxacin and to a lesser extend of solithromycin activity towards young to mature naïve and induced pneumococcal biofilms made by reference strains (ATCC49619 and R6) and AECB clinical isolate N6 were confirmed by evaluating the modulations of the activity of another fluoroquinolone, levofloxacin, in the same conditions as described in the previous paper. These results are non-published data but are illustrated in a summary graphical fashion (see Figure 35) with the comparison between levofloxacin maximal efficacies (E_{max}) values obtained towards bacterial survival and biomass in the same biofilm types.

Maximal efficacies are expressed as percentages of 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 (upper panel, [A]) , R6 (middle panel, [B]) and N6 (lower panel, [C]). Values were calculated as means ± SEM 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. 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 35


A
E_{max} (% reduction in signal *vs.* control) - ATCC49619
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
B
E_{max} (% reduction in signal *vs.* control) - R6
viability
biomass
2 days
11 days
naive
induced
CTR
SAL
SAL+ZAN
SAL+IPR
IPR
C
E_{max} (% reduction in signal *vs.* control) - N6
viability
biomass
2 days
11 days
naive
induced
CTR
SAL
SAL+ZAN
SAL+IPR
IPR

5. Set up of a new viability assay based on the pneumococcal NADH oxidase

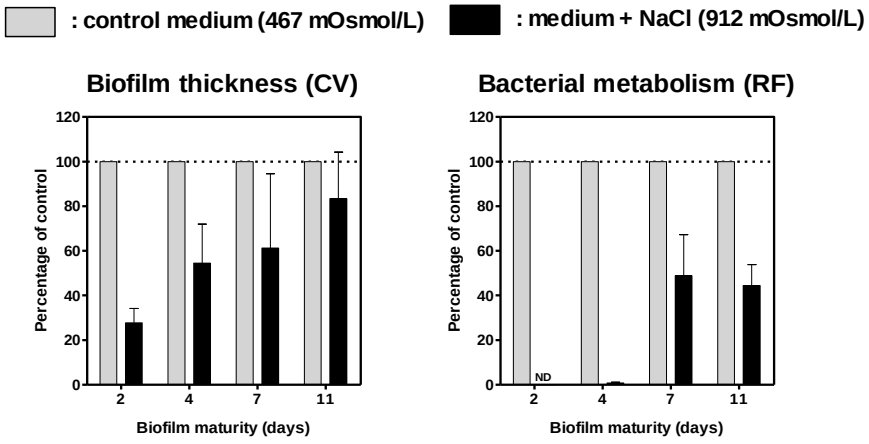
5.1. Introduction

In previous chapters, bacterial viability was quantified using the resazurin viability assay. This method is cheap, accurate and easy to quantify and follow the cellular metabolic activity through measures of resorufin fluorescence. Because metabolism and viability are in most of cases well-correlated, this assay is more and more used to evaluate the bacterial viability within biofilms. However, such as illustrated in Figure 34, this method is also characterized by a poor sensitivity, since the lower limit of quantification is approximately 10^{4-5} colony forming units (CFU)/mL. Similar observations are also reported in the literature for *in vitro* biofilms made by other pathogens (Van den Driessche F. *et al.*, 2014). Moreover, the rate of the resazurin reduction into pink and resorufin differs from one bacterial species to the other and is very long for young pneumococcal biofilms, such as shown in Table 5. Additionaly, some experiments performed during this thesis have shown that this assay does not enable to distinguish bacterial death from dormant cells (slowed metabolic function). This limitation is illustrated here below, in Figure 36, with results of an osmotic stress experiment.

5.1.1. Demonstration of S. pneumoniae adaptation to osmotic stress in biofilms

Figure 36 illustrates an experiment performed on ATCC49619 naïve biofilms grown under control conditions or in medium supplemented with sodium chloride in order to create an osmotic stress. The biofilm thickness was quantified overtime through crystal violet staining and the bacterial metabolism in sessile bacteria with the resazurin assay. Results are expressed in percentages of values obtained under control conditions for crystal violet (CV) absorbances and resorufin (RF) fluorescence.

Figure 36



Comparison of the biofilm thickness and metabolic activity in naive *in vitro* ATCC 49619 biofilms of 2, 4, 7 and 11 days of maturity grown under control conditions (caMHB supplemented with 5% of lysed horse blood and 2% of glucose; osmolarity: 467 mOsmol/L; grey bars) or in osmotic stress conditions (caMHB supplemented with 5% of lysed horse blood, 2% of glucose and with 2% of NaCl; osmolarity: 912 mOsmol/L; black bars). Results of crystal violet (CV) absorbances or resorufin (RF) fluorescence are means ± SD of 2-4 independent determinations. Results are expressed in percentages of the mean value measured for each maturity stage under control conditions and set to 100 percents. ND: no signal detected; the fluorescence signal is lower than the limit of detection.

For 2- and 4-days old biofilms, it appears that the osmotic stress induces a bacterial switch from the active metabolic state to an inactive or dormant state. Indeed, the right panel, illustrating the bacterial metabolic activity through the reduction rate of resazurin into fluorescent resorufin, shows that, for these two maturity stages, no or very little fluorescence signals were detected. Despite of this, when biomass is considered (left panel), we can observe that some matrix production occurs in young biofilms, even if the amounts produced are significantly lower than for biofilm growth under control conditions. This demonstrates that the osmolarity-mediated loss of resazurin reduction may not be attributed to bacterial killing but well to a decreased metabolic function.

For mature biofilms (7- and 11-days old), a regain of bacterial matrix production was observed for biofilms grown in presence of NaCl with crystal violet absorbances reaching the same values as for biofilm development in control medium. Similarly, the fluorescence signals increased at old maturity stages, by comparison with young biofilms but without reaching 100% of reduction.

This experiment translates the bacterial capacity of adaptation with a good ability to undergo the metabolic activity to survive to harvest environmental conditions. These cells, living in a dormant state, are defined as "persisters" in the literature. This particular state is highly present and described for bacterial growth within biofilms and favors the resistance to antimicrobials (Simoes, 2011). Secondly, this study highlights once again the protective role played by the matrix. Indeed, Figure 36 shows that when the amount of matrix produced reach a sufficient thickness threshold to protect sessile cells from the osmotic stress, the metabolic function increases.

However, this osmotic stress experiment shows that reduction of resazurin sodium salt into resorufin may be affected in the case of bacterial death but also, in the case of dormant cells (slowed metabolic function), leading to the impossibility to distinguish these two bacterial states and an antibiotic bactericidal activity from a bacteriostatic one.

For this reason, we tried to develop a new enzymatic method for bacterial viability quantification in pneumococcal planktonic cultures and biofilms that addresses weaknesses of the resazurin viability assay. For that purpose, the literature describing the different enzymes of *Streptococcus pneumoniae* was carefully examined and finally, we decided to focus our attention on the cytoplasmic NADH oxidase. The set-up of the viability assay based on the activity of this enzyme is outlined in detail hereafter, as well as the results of antibiotic killing activities obtained with this method, in comparison with the resazurin assay and CFU counting.

5.1.2. Streptococcal NADH oxidases

Two types of NADH oxidases (NOX) are actually described for streptococci. The bacterial species for which they are the most characterized is *Streptococcus mutans*. For this pathogen, NOX-1, present at the membrane surface plays an adhesin role and participates, through the reduction of O_2 into H_2O_2, in the respiratory process and oxidative stress (Higuchi *et al.*, 2000;Yamamoto *et al.*, 2006). This reaction is accompanied by the oxidation of the co-factor NADH ($+H^+$) into nicotinamide adenine dinucleotide (NAD^+) and the impairment of NOX-1 oxidative activity leads to a major decrease in cellular growth. This phenomenon mainly occurs for early stages of biofilm development when the protein is mainly involved in bacterial adhesion to a support. The cytoplasmic type of NADH oxidase present in *S. mutans* is called NOX-2. This enzyme is involved in the glucidic metabolism by allowing the O_2 reduction into H_2O and the loss of its activity leads to an increase of the intracellular lactic fermentation (Higuchi *et al.*, 2000;Yamamoto *et al.*, 2006).

The implication of pneumococcal NADH oxidases in these two same processes has already been mentioned in the literature (Chapuy-Regaud *et al.*, 2001). However, at the opposite of *S. mutans*, no specific nomenclature exists, to our knowledge, to distinguish the cytoplasmatic enzyme from its membrane counterpart in *S. pneumoniae*. Moreover, the NADH oxidase present at the pneumococcal surface is considered as a major virulent factor (Muchnik *et al.*, 2013).

Based on this description of NOX enzymes, we decided to set-up a new enzymatic viability assay also capable to distinguish a bactericidal effect from an inhibition of bacterial growth observed with bacteriostatic compounds. These two types of activities can be found among the different antibiotic classes active against *S. pneumoniae*, for example, with fluoroquinolones (e.g. moxifloxacin) presenting a bactericidal effect and macrolides (e.g. clarithromycin) being mainly bacteriostatic.

5.2. Principle of the NADH oxidase-based assay

The principle of this new assay is the following: when the bacterial membrane is lysed, the intracellular content, including the cytoplasmic NADH oxidase, will be released into the extracellular compartment. If NADH is thereafter added in culture plates containing a bacterial suspension or a biofilm treated with such a lysing agent, we will observe NADH consumption much more important than for bacterial incubation with compounds that do not affect the membrane integrity, such as bacteriostatic antibiotics. The consumption of NADH, oxidized into NAD^+ by the NADH oxidase, is followed and quantified by spectrophotometric measures of NADH absorbances at 339nm. Figure 37 illustrates the difference in membrane integrity and NOX release observed with a bactericidal versus a bacteriostatic antibiotic, the oxidation of NADH into NAD^+ by the NOX enzyme and their absorbance spectrum.

5.3. Methodology

To set-up this new assay, two different models were used: 24-hours planktonic cultures, made with fresh colonies grown on agar plates and resuspended in caMHB supplemented with 5% of lysed horse blood , and 2-days old biofilms obtained using the same procedure as described before. Both models were treated during 24 hours with a gradient of moxifloxacin or clarithromycin concentrations and two controls were used in parallel: culture medium without antibiotic (negative control) and a solution of daptomycin (Cubist Pharmaceuticals, 2µg/mL in culture medium with a calcium concentration fixed at 50mg/L) used as positive bactericidal control. This lipopeptide antibiotic is characterized by a membrane-destabilizing power leading to complete lysis and was used at a concentration highly bactericide for *S. pneumoniae* (Restrepo *et al.*, 2003). In the case of this assay, daptomycin was preferred as positive control to sodium dodecyl sulfate, used in the resazurin viability assay, because it offers the advantage of not affecting the activity of the released cytoplasmic NADH oxidase.

The bacterial viability was assessed after this incubation time using in parallel (i) CFU counting and the resazurin and NADH oxidased-based viability assays for planktonic cultures and (ii) only the resazurin and NADH oxidased-based viability assays for biofilms. The reasons for this was that CFU counting is less evident to perform on sessile bacteria because of (i) the difficulty of disrupting the whole biofilm with sonication and recovering all the cells, and (ii) because these cells may remain partially aggregated such as within the extracellular matrix and, therefore, give underestimated bacterial counts after platting.

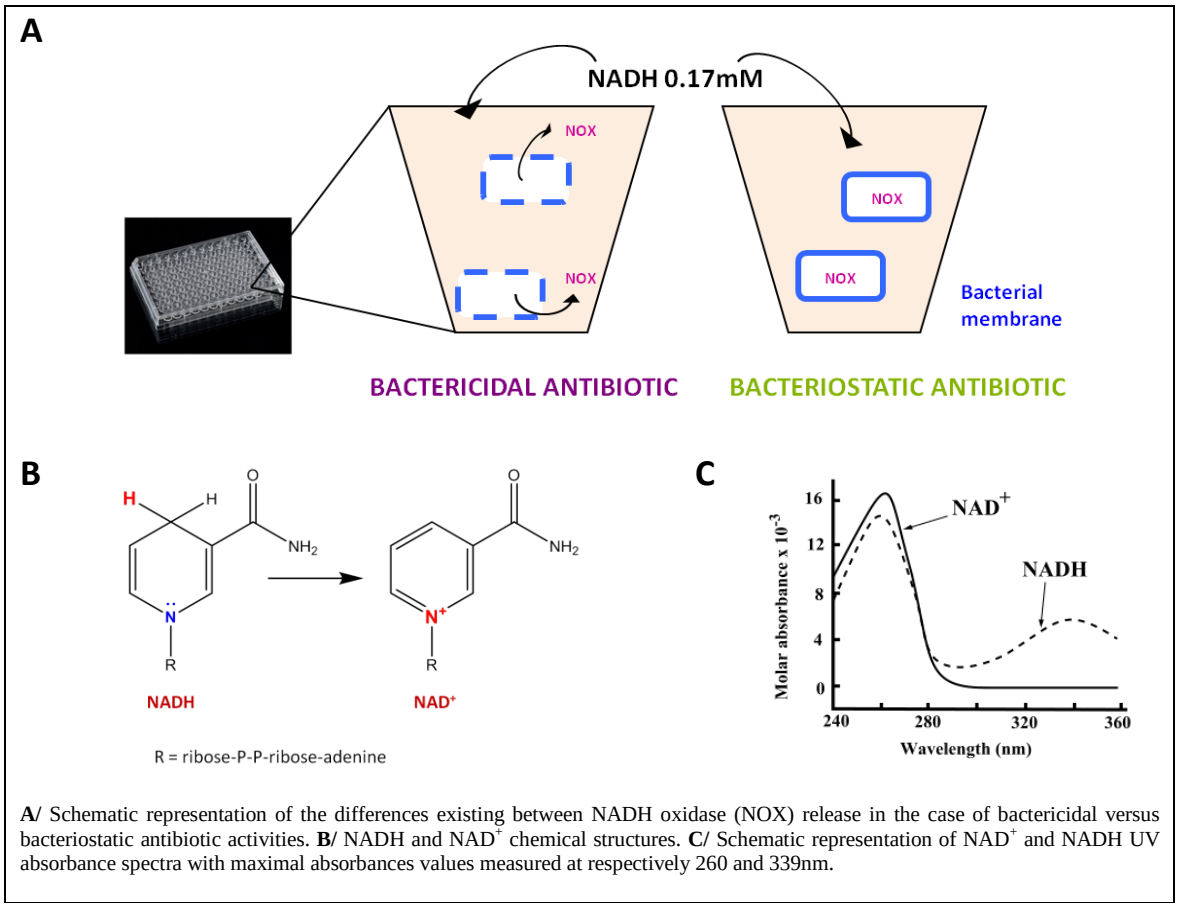
The different steps followed for spectrophotometric quantification of bacterial viability are described here below:

1) After the 24 hours of incubation with the antibiotic gradient, 100µl of NADH 0.17 mM aqueous solution were added to (i) 100µl of treated planktonic culture or to (ii) 96-well plates containing sessile bacteria from a biofilm treated with the antibiotics and washed with phosphate saline buffer.

2) NADH absorbance (λ339nm) was recorded every minute, during 30 minutes, at 37°C.

3) For planktonic cultures, NADH oxidation into NAD^+ was faster than for sessile 2-days old biofilms, ensuring that maximal NAD^+ amounts were reached after 1 minute in the first model and after 30 minutes in the second. Results of antibiotic activity were expressed in percentage of the negative control, after having subtracted OD values measured for the positive control, such as for the resazurin viability assay.

Figure 37

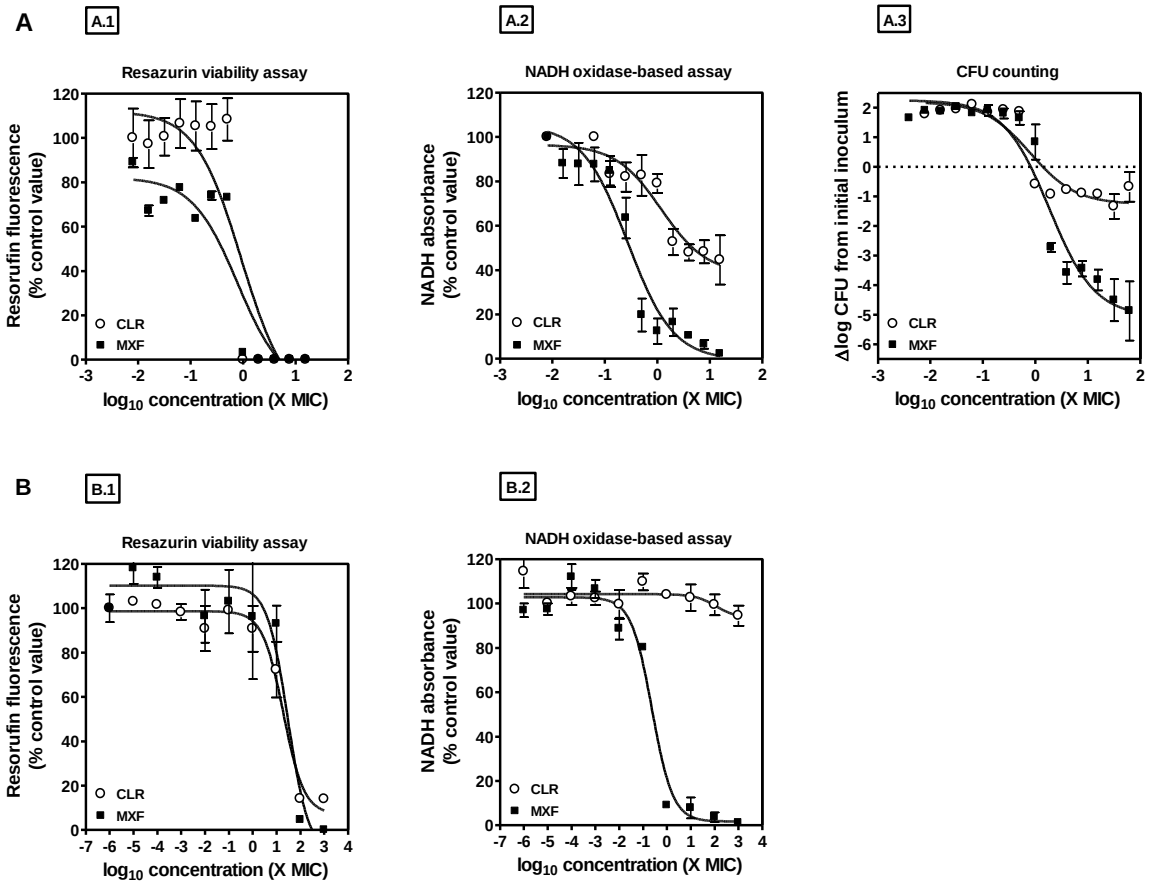


A/ Schematic representation of the differences existing between NADH oxidase (NOX) release in the case of bactericidal versus bacteriostatic antibiotic activities. **B/** NADH and NAD^+ chemical structures. **C/** Schematic representation of NAD^+ and NADH UV absorbance spectra with maximal absorbances values measured at respectively 260 and 339nm.

5.4. Results

Results of antibiotic activity on survival obtained, through pharmacodynamic studies, on planktonic cultures and 2-days old biofilms made with the reference strain ATCC49619 and evaluated using the three methods previously described are presented in Figure 38 and Table 6. These results were presented in the form of a poster (see Figure 39) at the 24th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in May 2014. A fifth paper combining the steps needed for the development of the NADH oxidase-based assay and the results of moxifloxacin and clarithromycin activities, on bacterial survival in planktonic cultures and young biofilms made by the reference strain ATCC49619 and some clinical isolates, obtained with this new method, in comparison with CFU counting and measures of resorufin fluorescence is in preparation.

Figure 38



Dose-response curves of moxifloxacin (MXF, full symbols) and clarithromycin (CLR, open symbols) activities on bacterial survival in planktonic cultures (A) and 2-days old biofilms (B) made with strain ATCC 49619, after 24hours of incubation with increasing concentrations of antibiotics. The ordinate shows the change in viability as a percentage of the control value (no antibiotic present) and measured by the decrease in resorufin fluorescence (A1, B1), in NADH absorbance (A2, B2) and in CFU (A3). All values are means ± SD of 2 to 6 independent experiments. When not visible, the error bars are smaller than the size of the symbols. Values of maximal efficacies (E_{max}), used as pertinent pharmacological descriptor of the curves, are presented in Table 6.

Table 6. Maximal efficacies (E_{max}) represented as percentages of reduction in bacterial viability

Antibiotic	24h planktonic cultures			48h biofilms	
	Resazurin assay (metabolism)	NOX assay (membrane integrity)	$\Delta \log_{10}$ CFU (cell enumeration)	Resazurin assay (metabolism)	NOX assay (membrane integrity)
Moxifloxacin (bactericidal)	**-113.3% ± 5.9 (A,a)**	**-100.4% ± 4.4 (A,a)**	**-3.8 ± 0.5 (A,b)**	**-110.7% ± 12.5 (A,a)**	**-98.3% ± 3.0 (A,a)**
Clarithromycin (mainly bacteriostatic)	**-121.0% ± 11.2 (A,a)**	**-61.7% ± 6.6 (B,b)**	**-0.7 ± 0.7 (B,c)**	**-92.9% ± 5.7 (A,a)**	**-6.5% ± 6.4 (B,b)**

Statistical analysis: values with different letters are statistically different from each other. Uppercase letters indicate, for each method, comparison between antibiotics (unpaired t-test, $p<0.05$) and lower case letters indicate, for each antibiotic, comparison between methods (one-way ANOVA with Tukey's post-test, $p<0.05$).

Setting up a NADH oxidase (NOX)-based assay to quantify the bactericidal activity of an antibiotic in planktonic cultures and biofilms of *S. pneumoniae*.

EP288

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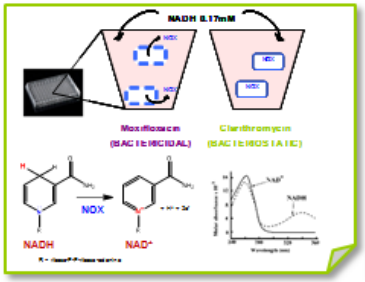
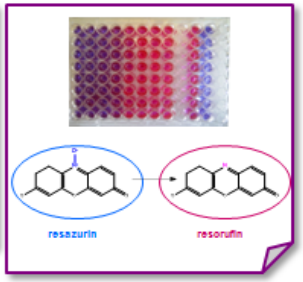
INTRODUCTION & AIM

- Measuring the release of a soluble cytosolic enzyme is widely used for cytotoxicity assay in eukaryotic cells, but is rarely applied to bacteria. Yet, it could help for the rapid screening of bactericidal antibiotics towards both planktonic cultures (PK) and biofilms (BF).
- In *S. pneumoniae*, NADH oxidase (NOX), a soluble flavoprotein involved in NADH reoxidation, is essential for virulence.[1] As NADH does not permeate through membranes,[2] its oxidation when added to the medium will be proportional to the amount of NOX release from lysed bacteria[3].
- The aims of the study were (i) to set-up a NOX-based assay using moxifloxacin (MXF) and clarithromycin (CLR) as representatives of bactericidal and bacteriostatic antibiotics, respectively,[4,5] and (ii) to compare its performance *vs.* CFU counting (PK) and resazurin reduction assay [6,7] (PK and BF).

MATERIALS & METHODS

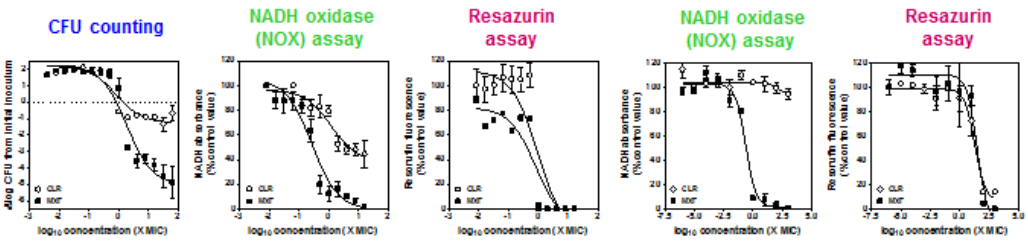
- **Bacteria** : *S. pneumoniae* ATCC49619; suspension (0.5 McFarland) in caMHB+ 5% lysed horse blood.
- **Models and antibiotic exposure** :
  - **Planktonic model** : incubation of bacterial suspension with antibiotics (0.03 to 8000 x MIC_{broth}) during 24h at 37°C, following the CLSI MIC microdilution protocol.
  - **Biofilm model** : 25µL of bacterial suspension added to 175 µL of medium (caMHB + 5% lysed horse blood + 2% glucose[7]) in 96-well plates and incubated during 48h at 37°C in 5% CO_2 atmosphere; biofilm incubated with antibiotics (10^{-6} to 10^{3} x MIC_{broth}) during 24h in the same conditions.
- **CFU counting** : enumeration of CFUs after plating on agar plates.
- **NADH oxidase-based assay** : measure of the reduction in NADH OD_{340nm} after 30 min.of incubation at 37°C (initial conc.: 0.17mM). Daptomycin (2 mg/L) is used as positive control (100 % mortality)
- **Resazurin assay** : measure of the increase in fluorescence signal (λ_{ex} 560nm; λ_{em} 590nm) of resorufin (pink, fluorescent) formed by resazurin (0.001%; blue, non fluorescent) reduction by living bacteria after 30 min (planktonic cultures) or 72 h (biofilms).





RESULTS

Characterization of moxifloxacin (MXF) and clarithromycin (CLR) activities (concentration-effect curves) on *S. pneumoniae* ATCC49619 planktonic cultures and biofilms, using 3 different assays



Antibiotic	Maximal changes in tested parameter				
	24h planktonic culture			48h biofilm	
	Δ log_{10} CFU (cell enumeration)	NOX assay (membrane integrity)	Resazurin assay (metabolism)	NOX assay (membrane integrity)	Resazurin assay (metabolism)
Moxifloxacin (bacteriolidal)	-3.8 ± 0.5 (A)	-100.4% ± 4.4 (A)	-113.3% ± 5.9 (A)	-98.3% ± 3.0 (a)	-110.7% ± 12.5 (a)
Clarithromycin (mainly bacteriostatic)	-0.7 ± 0.7 (B)	-61.7% ± 6.6 (B)	-121.0% ± 11.2 (A)	-6.5% ± 6.4 (b)	-92.9% ± 5.7 (a)

Statistical analysis : values with different letters are statistically different from each other (unpaired t-test, $p<0.05$). uppercase letters: planktonic culture; lower case letters: biofilm

- **the resazurin-assay did not distinguish between bactericidal and bacteriostatic antibotics (both caused a concentration-dependent reduction in signal that reached a minimum at low multiples of the MICs for planktonic cultures as well as in in biofilms);**
- **conversely, in both planktonic cells and biofilm, the NADH oxidase assay allowed for a unambiguous differentiation between bactericidal (MXF) and bacteriostatic (CLR) antibiotics.**

SUMMING UP & CONCLUSIONS

- We set up a new sensitive method allowing for the rapid quantification of antibiotic bactericidal and bacteriostatic activities in pneumococcal planktonic cultures and biofilms.
- As this NADH oxidase-assay can be completed in 30 min and is amenable to performance in various media, it may offer advantages for the rapid screening of antibiotic activities in different culture conditions.
- Application to other bacterial species warrants further investigation.

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This poster will be made available for download after the meeting at: http://www.facm.ucl.ac.be/posters.htm

Figure 39

5.5. Discussion

In both models and for the three different methods of viability quantification, killing activities were represented by sigmoid dose-response curves and increased with the antibiotic concentration. In planktonic cultures and 2-days old biofilms, results obtained with the resazurin viability assay are showing that high moxifloxacin and clarithromycin concentrations inhibit almost completely the resazurin reduction. This is represented by bacterial residual viability values close to 0%, by comparison with the negative control. Therefore, the distinction between the bactericidal versus bacteriostatic properties of the two antimicrobials is impossible.

At the opposite, the NADH oxidase assay allowed to make the difference between these two types of antimicrobial activities. With this method, moxifloxacin was shown as killing more than 80% of planktonic and sessile cells at its MIC in broth. Clarithromycin activity on survival was markedly less important with only 60% of decrease in viability signal for planktonic cultures and no effect on biofilms.

The difference between the bactericidal and bacteriostatic effects provided by the NADH oxidase-based assay for bacteria incubated during 24 hours with moxifloxacin and clarithromycin, was validated with bacterial counts in the planktonic model (see A3). Bactericidal activity is defined as a ≥ 3 $\log_{10}$ decrease in CFU/mL, whereas bacteriostatic activity is defined as a <3 $\log_{10}$ decrease in CFU/mL (Pearson *et al.*, 1980). The rate and extent of killing were determined by plotting viable count (change in $\log_{10}$ CFU/mL t_{24h}-t_{0h}) against antibiotic concentration ($\log_{10}$ MIC_{broth}). The lower limits of detection observed for moxifloxacin and clarithromycin were respectively -4.9 and -0.7 $\log_{10}$ CFU/mL, demonstrating that moxifloxacin kills well bacterial cells, when clarithromycin only inhibit their multiplication.

Concerning the comparison of results provided by this new assay for the planktonic (A2) versus sessile model (B2), we can notice that the difference between moxifloxacin and clarithromycin maximal efficacies is much more marked for sessile bacteria than for planktonic ones. Yet, there is evidence that clarithromycin is bacteriostatic in both conditions (see A3). Therefore, we can hypothesize that NADH consumption observed in planktonic cultures and translated by a decreased absorbance is not related to bacterial cell lysis but that the membrane NADH oxidase is responsible, in this model, of some NADH consumption. This activity of the cytoplasmic enzyme does not pose bias problems in the sessile model because, during biofilm development, this protein plays mainly a role in bacterial adhesion and, in this situation, its oxidase activity is highly decreased (Derr *et al.*, 2012 ; Nguyen *et al.*, 2002).

Concerning the limits of the NADH oxidase-based assay, we have to mention that, the method, at its current stage of development, is only applicable to young biofilms (< 4 days of maturity) because matrix thickness interferes with spectrophotometric readings when its amount becomes too important. In terms of crystal violet (CV) absorbance, it represents biofilms with a CV optical density higher than 4. However, further perspectives to deal with this problem may perhaps be a biofilm treatment with some agent inducing the matrix disassembly, before proceeding to NADH addition in culture plates. This means finding a compound acting on the matrix without affecting the activity of the cytoplasmic NADH oxidase released within the EPS from lysed sessile bacteria.

Discussion

1. Main findings of this work and clinical interest of this study

1.1. Pneumococcal resistance to antibiotics and serotype/serogroup

Through the isolation and characterization of *Streptococcus pneumoniae* clinical strains collected from AECB patients living in different Belgian areas, we assessed the pneumococcal susceptibility profile to antibiotics in our country during the last 8 years and purchase the investigations of the laboratory concerning the incidence of pneumococci antibiotic resistance. Antibiotics investigated belong to classes active against *S. pneumoniae* and currently prescribed around the world to treat bacterial exacerbations of chronic bronchitis. The main findings were that β-lactams (amoxicillin, cefuroxime and ceftriaxone) and fluoroquinolones (levofloxacin and moxifloxacin) maintain a good activity against *S. pneumoniae*. This is reassuring, taking into account that Belgian recommendations are promoting the use of amoxicillin, alternatively or not with moxifloxacin to treat AECB patients (Belgian Antibiotic Policy Coordination Committee (BAPCOC), 2012; Van Bambeke *et al.*, 2007). Concerning the use of macrolides, our MIC results showed a huge rate of resistance, supporting the view of their inappropriateness. However, ketolides demonstrated a very high level of activity and, solithromycin, currently in phase 3 of clinical development, offers new hopes for patients' management, principally because of its good killing activity against resistant strains, also demonstrated in other studies (Farrell *et al.*, 2010), and its lower toxicity, compared to telithromycin and explained by a modified structure (Bertrand *et al.*, 2010). This ensures that this molecule might be included in future therapeutic guidelines as an alternative to amoxicillin.

Concerning serotyping analyses, our study revealed that most of strains were at least partially covered by existing vaccines. This brings the question of the rational of partial vaccination (with vaccines covering only some pneumococcal serotypes but not all of them). Our study also brought the useful information that some serotypes/serogroups are more associated with resistance to all antibiotic classes or specifically resistant to some antimicrobials. This introduces a new concept in terms of therapeutic choice decision based on detected surface antigens in the order to administrate the most adequate anti-infectious treatment with the possibility of customizing it to the causal pneumococcal strain. Recent studies, also describing higher resistance rates for some serotypes, help to better understand the underlying mechanisms by having investigated the implication of the Pneumococcal surface protein A (PspA), a virulence factor. This protein contains five domains including a signal peptide, an alphahelical charged region, a proline-rich domain, a choline-binding domain and a C-terminal amino acid tail. Depending on the nucleotide sequence of the alpha-helical region, PspAs are classified into three families (A1, A2 and A3). In 2013, Hotomi and colleagues published some data showing, on one hand, that a serotype may be more resistant to β-lactams and/or macrolides than others and, on the other hand, that a same serotype may express A1, A2 or A3 with a similar prevalence for A1 and A2

and a less frequent expression for A3 (Hotomi *et al.*, 2013). Based on these findings, authors grouped their strains according to the types of PspA expressed and they could demonstrate that the serotype was not a direct reflect of the resistance pattern but that resistance seems to be more associated with PspA families, A3 being the more susceptible. Because A1 and A2, associated with higher resistance to betalactams and macrolides, are more frequently expressed in some serotypes than A3, it gives the illusion that capsular polysaccharides are reflecting the susceptibility of the strain. However, it has been shown that if the same serotype is expressing A3, the strain is well susceptible to the two antibiotic classes previously mentioned (Hotomi *et al.*, 2013). Therefore, it seems more accurate to conclude that, in most of cases, serotype reflects the type of PspA expressed and that this parameter is, for reasons not yet firmly established, closely associated with the resistance pattern.

1.2. Characteristics of patients suffering from acute exacerbations of chronic bronchitis

The collection and analysis of the medical data from AECB patients referred to the hospital between November 2010 and May 2013, allowed us to better know what their main characteristics are. Concerning demographic factors, the study revealed that most of patients were men and presented different types of co-morbidities, hypertension being the most frequent followed by psychiatric disorders, alcoholism, cancer and diabetes. These results are consistent with what is described in the literature for COPD patients (Sin *et al.*, 2006 ; Sisson, 2007, Koskela *et al.*, 2014). These observations raise once again the question of the causal relationship existing between respiratory impairment and the development of co-morbidities and the question of the importance of early COPD diagnostic.

1.3. *In vitro* naïve and induced biofilms: models, methods and pharmacodynamics studies

Biofilms studies in our laboratory started in 2010, with the beginning of this thesis. To investigate the antibiotic activity towards *in vitro* sessile pneumococcal cells, the first step required was having an adequate model.

Therefore, we developed two types of pneumococcal biofilms with the aim of having models mimicking primary infection (naïve biofilms) and secondary colonization (induced biofilms). This had been achieved by using respectively fresh pneumococcal colonies or pneumococcal cells collected in the supernatant of a parent biofilm. This approach for studying *in vitro* biofilm development was totally new, when looking to the actual literature or to papers existing at the beginning of this project. Indeed, in 2010, pneumococcal biofilm *in vitro* models remained small in number and were not very well developed. In most of cases, only young maturity stages (1-6 days) had being investigated. However, our studies revealed that these maturity stages are not sufficient to reach the maximal biomass level. Biofilms are considered to play an important role in bacterial persistence, partly because of the barrier role played by the matrix, decreasing the antibiotic activity (Simoes, 2011). In the context of recurrent and deep infections, we thought that it would be interesting to get old maturity

stages with huge amount of biomass. To this aim, we first performed biofilm culture up to day 11, from fresh bacterial colonies grown on agar plates (naïve model).

Using this biofilm type, we could continue our studies about pneumococcal parameters (origin, serotypes/serogroup, antibiotic efflux phenotype and MIC).

These studies offered new and original characterizations of pneumococcal infections occurring in COPD patients and involving bacterial growth within biofilms. Main results were that all serotypes/serogroups do not have the same ability to produce biomass. This may also explain why some pneumococcal infections are more difficult to eradicate, independently on the recurrent character of AECB episodes. Our results also demonstrate that the rate of matrix production was not linked to the strain susceptibility profile and neither to antibiotic efflux. This differs from what is described for other respiratory pathogens such as *Pseudomonas aeruginosa* for which biofilm production and efflux are negatively correlated to avoid the extrusion of essential quorum sensing signaling molecules (Hurley *et al.*, 2012 ; Lamarche and Deziel, 2011 ; Sanchez *et al.*, 2002 ; Pearson *et al.*, 1999) and illustrates how the bacterial susceptibility profile to antibiotics may be affected or not, depending on the type of quorum sensing pathways involved in biofilm formation. Moreover, our work showed that the severity of bronchial obstruction and, therefore, airways oxygenation in COPD patients did not influence the biomass production by clinical isolates, at the opposite of patients' medication and especially ipratropium, a muscarinic antagonist, inducing matrix disassembly and so, allowing reaching almost 100% for maximal antibiotics killing efficacies.

Matrix disassembly is a natural process, already described for biofilms made by other bacterial species such as *S. aureus* (Boles and Horswill, 2011) and involved in tissue colonization through the release of bacterial cells and their return to the planktonic state (Vlastarakos *et al.*, 2007).

Because it was possible that these cells, coming from a "parent biofilm structure" have been stimulated for matrix production through quorum sensing signaling, but that this aspect had never been explored in studies and models prior to ours, the second biofilm model that we developed was made using cells released in the supernatant of an existing *in vitro* biofilm and inoculated to new culture plates to restart a new cycle of biofilm development until a new mature structure. This model was new in 2010 and demonstrated that induced bacteria produced more matrix than naïve ones, grown on agar plates. Additionaly, our observations were comforted by other authors. For instance, in 2011, Trappetti and colleagues confirmed that mice infected by sessile cells, coming from a parent structure, are presenting more biofilm matrix formation in lungs and brain tissues than their counterparts infected by the same strains but in a fresh planktonic state. This was explained by the fact that expression of genes *ciaR* and *ciaH*, involved in colonization and adherence to human lung cells, was different between sessile and planktonic bacteria *in vivo* and *in vitro* (Trappetti *et al.*, 2011a).

Good correlations between *in vitro* static and *in vivo* biomass production have already being described in the literature, legitimizing the use of static inert surfaces for this type of research.
Concerning the use of polystyrene supports for *in vitro* biofilm growth, several recent studies have shown that despite the fact that interactions between prokaryotic and eukaryotic cells are not mimicked in experiments performed on static inert supports and, consequently, that several aspects influencing the infection outcome *in vivo* (e.g. the modulation of quorum sensing by cytokines secretion,…), are not reproduced in our experiments, it appears that the pneumococcal propensity to form biofilm *in vivo* closely correlates with the degree of biofilm formation in microplates (Blanchette-Cain *et al.*, 2013; Vidal *et al.*, 2013; Bowler *et al.*, 2014). These observations can be explained by the fact that the factors involved in this process do not all have the same weight in determining *S.pneumoniae* biofilm formation (Blanchette-Cain *et al.*, 2013).

For instance, some adhesins and virulence factors (e.g. CbpA, LytA, pneumolysin,…) participate to the biofilm development but are not crucial (Blanchette-Cain *et al.*, 2013; Marks *et al.*, 2012). Some changes in their level of expression between *in vitro* and *in vivo* sessile pneumococci would therefore not lead to significant differences in biomass amounts. This hypothesis has already been confirmed with mutants deficient for each of these proteins (Marks *et al.*, 2012). However, this cannot be generalized to all the adhesins-types. Indeed, results published in 2010 by Sanchez and colleagues and focused on the pneumococcal serine-rich repeat proteins (PsrP), showed that depending on the type of PsrPs expressed, bacterial attachment to abiotic (polystyrene microplates) or viable surfaces (tissues or cells layers) can be different (Sanchez *et al.*, 2010).

Concerning the quorum sensing signaling, in 2006, Oggioni and colleagues published a good comparison of the role of the quorum sensing competence stimulating peptide (CSP) in biofilm formation on polystyrene microplates and in a murine model. They observed that *in vitro* biofilm growth in a medium supplemented in CSP or administrating the peptide to mice increased (i) cell attachment to plastic and biofilm production *in vitro* and (ii) biofilm formation and bacterial virulence *in vivo* with a higher rate of mice death (Oggioni *et al.*, 2006).

Additionally to the development of *in vitro* biofilm models, we adapted or developed viability assays in order to quantify bacterial survival within EPS. Indeed, existing routine methods allowing viability quantification within the matrix of biofilms made by *S. pneumoniae* were also not sufficient. CFU counting or microscopy were the two methods frequently reported in the literature at the beginning of this thesis and present respectively the inconvenient of underestimating the number of bacterial cells through uncompleted matrix discard and bacterial killing related to sonication, before bacterial platting on agar plates and the cost-related inconvenient of confocal microcopy making this technique poorly adapted to the completion of a large number of long-term experiments.

For all of these reasons, we decided to adapt the resazurin viability assay, described in the literature for the quantification of eukaryotic cell survival (O'Brien *et al.*, 2000 ; Perrot *et al.*, 2003) or the viability in *S. aureus* biofilms (Tote *et al.*, 2008b), to young and very mature naïve and induced pneumococcal biofilms. This method was mainly used to quantify bacterial viability after antibiotic treatment and the metabolic activity in planktonic and sessile pneumococcal cells. However, because it does not offer the ability to distinguish bacteriostatic from bactericidal effects or dormant cells from killed bacteria, we tried to set-up a new assay based on the activity of the pneumococcal cytoplasmic NADH oxidase. This method is totally new in our laboratory and also in the literature. Because this method is also probably easy to adapt to other bacterial species, we hope that it would be useful to other researchers working on other bacterial species and open new doors in terms of applications ideas.

Concerning pharmacodynamic studies of antibiotic activity, the main findings of this work were that antibiotics were globally less active against biomass than bacterial survival. A loss of activity towards sessile cells occurred over the increase of biofilm maturity and was related to an increasing matrix effect. The two most active antibiotics in our models were solithromycin and, especially, moxifloxacin. This is coherent with current Belgian therapeutic guidelines recommending the administration of that fluoroquinolone, in alternation with amoxicillin, in the case of frequent exacerbations episode occurring in COPD patients (Van Bambeke *et al.*, 2007; Belgian Antibiotic Policy Coordination Committee (BAPCOC), 2012). This molecule is also described in the literature as more active than the classic β-lactam therapy for this type of infections (Wilson *et al.*, 2004 ;Wilson *et al.*, 2012).

Moreover, the antibiotic activity on sessile pneumococcal cells could be highly restored, even in the case of very old maturity stages, if biofilms were grown in the presence of bronchodilators. The more important synergistic effects were obtained with ipratropium, an agent demonstrated in our study as able to induce an almost complete biofilm disassembly, probably through an inhibition of adhesines (Maestro *et al.*, 2007). The interest of this work was that all the non-antibiotic drugs added in biofilm culture medium were used at human therapeutic concentrations. Our study, also demonstrated that salbutamol, a short acting β2-agonist increases the activity of the Neuraminidase A, an enzyme described in the introduction as a main virulent factor, mainly because of its implication in biofilm formation and the remodeling of its three dimensional structure (Trappetti *et al.*, 2009; Parker *et al.*, 2009). Through this effect, salbutamol also improved antibiotic killing activity towards cells living within young naïve and induced biofilms. These results offer new perspectives in terms of therpauetic management with drugs associations and also help to better characterize how pneumococcal virulent factors may be target specifically with human drugs.

2. Limitations of this work

2.1. Epidemiological study

One of our objectives was to obtain an overview of the pneumococcal antibiotic susceptibility profile in Belgium and, more specifically, to examine if the incidence of resistance is (i) higher in the case of recurrent infections (*e.g.* AECB) than in the case of acute infections (*e.g.* community acquired pneumonia, …), (ii) more frequent in some geographic areas than others and (iii) related to some patients' demographic factors and physiopathological characteristics.

In that context, our work was focused on a collection of *Streptococcus pneumoniae* causing acute exacerbations of chronic bronchitis. This collection was assembled through collaborations with Belgian hospitals located in different areas of the country. Obviously, this meant collecting only strains coming from severe patients (in most of cases hospitalized or requiring a prior medical consultation). Therefore, the collection will be biased from isolates causing only mild infectious episodes and for which patients were treated at home.

Moreover, because our study was also focused on potential relationships existing between strains' characteristics and patients' medical data, for practical reasons, only a small number of patients, and corresponding *S. pneumoniae* isolates, were retained. Concerning the resistance profile, our results corroborate with the findings of other national and international studies (Lismond *et al.*, 2012 ; Vanhoof *et al.*, 2010 ;Wilson *et al.*, 2012). It is therefore likely that restarting similar studies with more isolates will not bring more useful information about the pneumococcal resistance profile. By contrast, concerning the link between the pneumococcal ability to produce biofilms and the strains' serotype or patients' medications, conducting experiments with hundreds of volunteers would be interesting to strengthen our data and going deeper in the understanding of underlying mechanisms.

2.2. Pharmacodynamic studies of antibiotic activity

2.2.1. Pneumococcal strains

In the same vein, only a small number of pneumococcal strains, including only 2 AECB clinical isolates, were used for pharmacodynamic studies of antibiotic activity towards young to very mature biofilms grown in control conditions or in the presence of bronchodilators. Our work confirmed the predominant modulatory effect played by the matrix in resistance to antibiotics and the implication of neuraminidase A in pneumococcal biofilm cohesion. By making the demonstration that human therapeutic doses of non-antibiotic drugs may help to deal with pneumococcal growth within biofilms we also opened doors for further investigations and new ideas to improve patient's management.

However, we cannot ignore the fact that differences in matrix composition exist in biofilms made by different strains of *S. pneumoniae* and that similarly, several forms of pneumococcal enzymes exist. Therefore, investigations of antibiotic activity on biofilms made by a large number of clinical isolates and developed in the presence of salbutamol, ipratropium and zanamivir and analogues of their respective drug classes would be interesting to assess the significance and impact of inter-strains variability on the biofilm development in the presence of bronchodilators and their response to antibiotics.

2.2.2. Biofilm models

Concerning the model used, this work was only performed using *in vitro* static biofilms. During the last ten years, several publications describing studies using *in vitro* models of biofilms made by *S. pneumoniae* were published. As we did, most of them are also using 96-well polystyrene microplates as support for growth (del Prado *et al.*, 2010 ; Tapiainen *et al.*, 2010 ; Kurola *et al.*, 2011 ; Roveta *et al.*, 2007 ; Lizcano *et al.*, 2010 ; Moscoso *et al.*, 2006 ; Munoz-Elias *et al.*, 2008 ; Trappetti *et al.*, 2009) because this method offers affordable testing of a large number of conditions at the same time by comparison with the use of a dynamic models (Allegrucci and Sauer, 2007 ; Donlan *et al.*, 2004 ; Vidal *et al.*, 2013). However, some growth modulating factors, such as shear forces occurring during biofilm development in dynamic *in vitro* models or *in vivo*, are absent in our models. This may constitutes a weakness and creates morphological differences between *in vitro* and murine pneumococcal biofilms models (Blanchette-Cain *et al.*, 2013).

Finally, the chemical composition of the extracellular environment composition also plays an important role in biofilm formation. In 2009, Trappetti and colleagues published some data showing how the concentration of a salivary sugar, sialic acid, modulates the number of colonies within biofilms in microplates and in the nasopharynx and lung tissues of infected mice (Trappetti *et al.*, 2009). In that context, we should not forget that the composition of medium used for biofilm growth is well adapted to pneumococcal proliferation *in vitro* but is very different from *in vivo* conditions. In that context, it appears that supplementations of the bacterial culture medium must be carefully studied to reproduce, as far as possible, conditions found in patients' airways. A good illustration of such improve is the use of an artificial sputum-like medium for biofilm development (Kirchner *et al.*, 2012).

2.2.3. Pharmacological evaluation of antibiotic acitivity towards sessile bacteria

In addition to differences between *in vitro* and *in vivo* bacterial growth conditions, the host inflammatory response occurring during *in vivo* infections could not be reproduced with the *in vitro* biofilm models used in this work. It has been reported in the literature that sessile bacteria, protected

by the extracellular biofilm matrix, resist host defenses cells (Keller and Costerton, 2009 ; Leid JG, 2009) and that quorum sensing signaling molecules downregulate the production of cytokines (Glucksam-Galnoy *et al.*, 2013). Because the immune response is a key determinant factor for the outcome of infectious diseases, it would be interesting to explore this aspect in more detail in order to determine which proportion of biofilm-related bacterial resistance observed in animals or in humans may be attributed to an impaired antibiotic activity and what is the real impact of bacterial growth within biofilms on the immune response.

Moreover, during pharmacodynamic studies performed on naïve and induced biofilms, gradients of antibiotic concentrations were used to obtain dose-effect curves but in each well, the antibiotic concentration tested remained constant throughout the 24h of incubation. This is also a significant difference between experimental conditions used in this work and the way of antibiotic administration used *in vivo* or to treat humans (except for antibiotic continuous infusion).

Additionnaly, the pharmacokinetic aspect of the antibiotic activity towards pneumococcal cells living within biofilms has not been studied during this thesis. The pharmacokinetics in focused on the fate of substances (in this case, antimicrobials) administred externally to a living organism and more specifically, studies the absorption, distribution, metabolisation and elimination of drugs. Limitations of antibiotic diffusion through the matrix have already been mentioned in the introduction. Further interesting studies would be to quantify the antibiotic distribution through measures of their concentrations at different depths of the biomass.

3. Perspectives

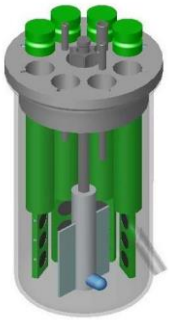
3.1. Dynamic *in vitro* biofilm models

Very soon, our laboratory will start new studies of antibiotic activity, using dynamic *in vitro* biofilms models. The "dynamic" or "flow displacement" models are open systems in which growth medium and waste-products are continuously added and removed, respectively. Based on the type of mixing occurring in these systems, devices are classified in two main groups: Continuous Flow Stirred Tank Reactors (CFSTR) and Plug Flow Reactors (PFR). In the first one, the feed rate is equal to the removal rate and therefore, the system works in "steady state" without changes in medium composition overtime and therefore, with identical conditions throughout the reactor. In the second type of devices, the mixing only occurs in a radial direction, following the direction of the flow and environmental conditions vary progressively throughout the reactor (Coenye and Nelis, 2010).

The CDC biofilm reactor developed by BioSurface Technologies and illustrated in Figure 40 is the most well-known CFSTR system. It consists of a glass vessel with a polyethylene top supporting eight removable polypropylene rods. Each of these rods can hold three removable coupons perpendicular to the rotating baffle and on which biofilms can form (Donlan *et al.*, 2004; Coenye and Nelis, 2010). A high shear or continuous flow of nutrients can be provided over the colonized surfaces through a central magnetic stirrer. In the future, this device will be used to investigate the biofilm formation on different surfaces. In a context of AECB, the first more relevant application would be pharmacodynamic studies of antibiotic activity towards pneumococcal biofilm developed on immobilized human respiratory epithelial or lung cells. This type of support has been described as allowing a faster and higher pneumococcal biomass production with less autolysis, by comparison with abiotic surfaces (uncoated polystyrene) (Vidal *et al.*, 2013).

Figure 40

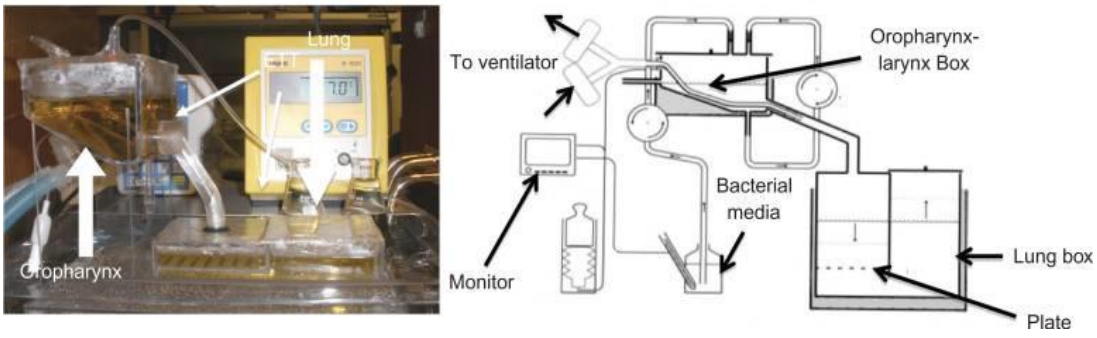
Two CDC biofilm reactor vessels coupled to a bottle with fresh medium. (Coenye and Nelis, 2010)



Detail of a CDC bioreactor *bioreactorhttp://www.biofilms.biz/biofilm-reactors*

Plug flow systems are good models of biofilm growth on catheters or endotracheal tubes. These devices are composed of a chamber filled with culture medium, simulating *in vivo* catheters flow conditions and containing several short and removable catheter segments on which biofilms can form when a bacterial suspension in added in the system (Machado *et al.*, 2012; Garcia *et al.*, 2010; Coenye and Nelis, 2010). A model of endotracheal tube system is illustrated on a pictorial fashion in Figure 41. The antimicrobial prophylactic and therapeutic activities of antibiotics can be easily quantified overtime with this system by taking out catheter segments at interval times for biofilm quantification. Moreover, this system is also more frequently used for experiments in which antibiotic concentrations are varying overtime, in order to mimick the fluctuations of human plasmatic levels. Such experiments would therefore response to a weakness of the studies performed during this thesis.

Figure 41



Model of an endotracheal tube (ETT) PFS system Machado *et al.*, 2012). The ETT is located in the oropharynx-larynx box and cut into small segments at the end of each trial to allow biofilm thickness quantification through crystal violet staining and enumeration of adherent bacteria through CFU counting.

3.2. Impact of non-antibiotic drugs on the activity of antimicrobials towards biofilms

Our work has revealed that non-antibiotic drugs such as bronchodilators improve the antibiotic activity towards biofilms by modulating the cohesion and thickness of their matrix. Because these effects were only obtained with only one representative drug of each bronchodilator class, it would be interesting to investigate the impact of biofilm growth in the presence of analogues of salbutamol and ipratropium such as the long-acting bronchodilators formotérol and tiotropium. Indeed, these molecules are recommended in priority for the treatment of severe bronchial obstruction occurring in COPD patients (GOLD, 2014).

Experiments mentioned in this manuscript describe the ability of salbutamol to enhance the neuraminidase activity and an almost complete loss of biomass mediated by ipratropium. Previous

results published in the literature have demonstrated that this last compound inhibits pneumococcal choline binding proteins, adhesins essential for bacterial attachment to supports and biofilm formation (Maestro *et al.*, 2007). In order to assess the impact of β2-agonists and muscarinic antagonists on the activity of the pneumococcal neuraminidase A and choline binding proteins respectively, it would be interesting to perform crystallization experiments. Co-crystallisation would allow the obtention of drug-enzyme complexes and provide interesting information about (i) the nature of amino acids and drugs subtituents participating to interactions, (ii) the binding strength of these drugs to the enzymes of interest, (iii) the complexes'stability formed and the nature of chemicals forces participating to these ligand-receptor interactions and, (iv) differences of affinity between these proteins and different bronchodilator analogues (Silva-Martin *et al.*, 2014).

3.3. Muti-species *in vitro* biofilms

Respiratory infections often involve simultaneous co-infections of patients' airways by different types of pathogens. On that basis, it would be interesting to produce biofilms made by different bacterial species in order to investigate the activity of antibiotics (in monotherapy or in combination) towards sessile bacteria. This situation is closer to the clinical reality, with a higher risk of horizontal transfer of resistance genes. Secondly, investigations of quorum sensing signaling within multi-species biofilms would perhaps also help to better understand why and how cooperation or competition are observed between different types of pathogens living within the same matrix (Burmolle *et al.*, 2014 ; Li and Tian, 2012). Mass spectrometry is currently the best method for quantification of quorum sensing mediators (Cheng *et al.*, 2014).

3.4. Phage therapy

This therapeutic antibacterial approach is based on the use of bacteriophages to remove bacterial biofilms. Concerning airways infections, this method has already shown good *in vitro* results towards biofilms and planktonic cultures made with *S. aureus* clinical isolates collected in nasal swabs of patients suffering from chronic rhinosinusitis with the advantages of preventing the emergence of bacteriophage-insensitive mutants (Drilling *et al.*, 2014). Their antibacterial activity in mediated by the production of depolymerases which degrade polysaccharides in biofilm matrix. However, this effect seems to be highly specific for host-derived polysaccharides and may therefore being limited in the case of multispecies biofilms (Pei and Lamas-Samanamud, 2014). Experiments performed with mono- or polyphage therapies would be interesting to define the real benefits of this approach in a context of polyinfections.

3.5. Study of the biofilm matrix composition

As perspectives, we would like to investigate at greater depths matrix changes in terms of composition during the different stages of the biofilm development. This will be made through enzymatic polysaccharides quantification and confocal microscopy. Indeed several fluorescent probes, specific for bacterial cells, extracellular DNA or different types of matrix polysaccharides allow the labeling, co-localization and quantification of biofilm constituents (Domenech *et al.*, 2013b ; [see Figure 20 for illustration]). Our objectives are to follow the biofilm composition during the different stages of the development and to better characterize the disassembly phenomenon observed in our experiments for old biofilm maturity stages or mediated by incubation with ipratropium. Moreover, the nature and amounts of matrix polysaccharides are known to vary among biofilms produced by different bacterial species. Therefore, the labeling and quantification of matrix constituents will also find some application in the case of multispecies biofilms.

3.6. *In vivo* biofilm models

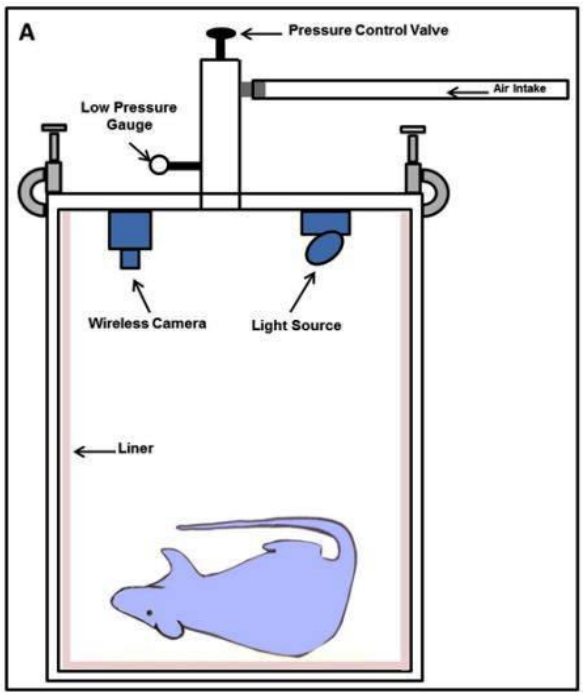
Extrapolation of *in vitro* results of antibiotic activity towards pneumococcal biofilms to the *in vivo* situation may not be directly made. However, during the last ten years, a high number of *in vivo* laboratory biofilm models made with a wide variety of species such as mice, rats, rabbits or dogs have emerged. These models can be used to study antimicrobials activity towards biofilms in a situation closer to the clinical reality with a special interest for drug pharmacokinetics and the interactions between sessile cells and the immune system.

Three main types of procedures are actually used to inoculate bacteria and induce biofilm formation *in vivo*. Namely, (i) a highly invasive route using the implantation of precolonised materials such as in osteomyelitis or deep airways infections models, (ii) a less-invasive inoculation through catheters used to induce, for example, urinary infections and finally, (iii) a non-invasive nasal inoculation, particularly used to induce ENT infections such as sinusitis or otitis media.

A sophisticated *in vivo* application of this last method to otitis media-related pneumococcal biofilms has been developed a few years ago by Chaney and colleagues (Chaney *et al.*, 2011). The originality of this model resides in the inoculation method and its ability to ensure otic colonization. This model and its principle are described in Figure 42.

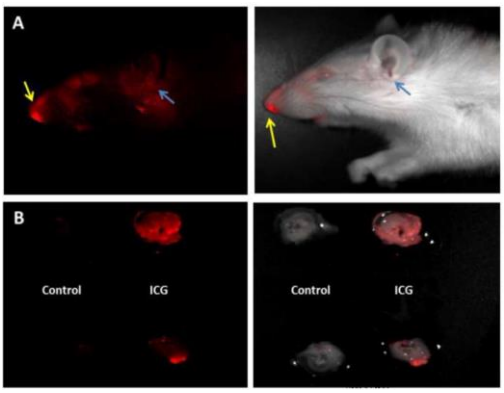
Figure 42

Prototype of Rat Pressure Chamber (Chaney *et al.*, 2011)

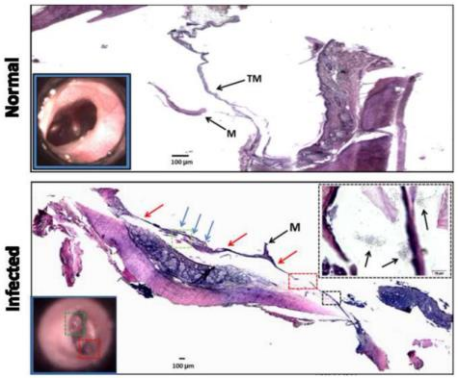


Schematic diagram of custom-modified pressure chamber

Full view of the completed chamber



(A) *In vivo* fluorescence darkbox imaging of an animal after ICG inoculation into the left nostril and pressurization. Yellow arrows indicate site of inoculation and blue arrows indicate presence of ICG in the ear canal. (B) *Ex vivo* fluorescence darkbox imaging of the TMs from inoculated (ICG) and control animals. In both figures, left images show fluorescence and right images show fluorescence signal overlay on brightfield images. In (B), the left and right TMs are shown at the top and bottom, respectively.



TOP: Histology (hematoxylin and eosin) of a TM from a normal (non-infected) animal showing a thin TM with no evidence of biofilm or bacterial colonies. Video otoscopy (inset lower left) shows a normal translucent TM. BOTTOM: Histology of a tympanic membrane from an infected animal. A biofilm (blue arrows) is present behind the TM (red arrows). The colored boxes on the histological section correspond to those in the video otoscope image (inset lower left). The histological section area in the black box is magnified and shown (inset upper right), revealing the presence of *S. pneumonia* colonies (arrows) located within and around the biofilm and TM. Abbreviations: M = malleus. TM = tympanic membrane.

Animals were first anesthetized and then nasally infected with a *S. pneumoniae* suspension (test group) or received a saline solution (control group). After inoculation, rats were immediately transferred to a custom-modified pressure chamber and orientated in a dorsal position to place the bacterial suspension closer to the nasopharyngeal entrance of the Eustachian tube. The chamber pressure was slowly increased to induce the diffusion of bacterial from nostrils to the middle ear. Bacterial inoculations were repeated every 4-7 days, for up to 7 months. To demonstrate the feasibility of the pressurization procedure and to ensure otic colonization, experiments were first performed using a 30µl of an Indocyanine Green (ICG; 20 mg/mL; 784nm absorbance) dye solution instead of the bacterial solution. The animal was pressurized, and following return to room pressure, was imaged *in vivo* using a darkbox (Maestro, CRI, Inc.) that permitted fluorescence excitation and imaging. *In vivo* imaging showed ICG fluorescence at the site of injection and in the ear canal. Over the course of the experiment, the tympanic membrane (TM) was observed for physiologic changes using a standard otoscope and animals were sacrificed at various time points to access the progression of biofilm formation. Following biofilm formation, the animals were sacrificed and the TMs were harvested for histological examination.

Conclusion

« Now, this is not the end. It is not even the beginning to the end.

But it is, perhaps, the end of the beginning. »

Winston Churchill

- London, November 10, 1942 -

Modulation of the antibiotic activity towards respiratory bacterial pathogens by co-medications: review of *in vitro*, *in vivo* and clinical studies

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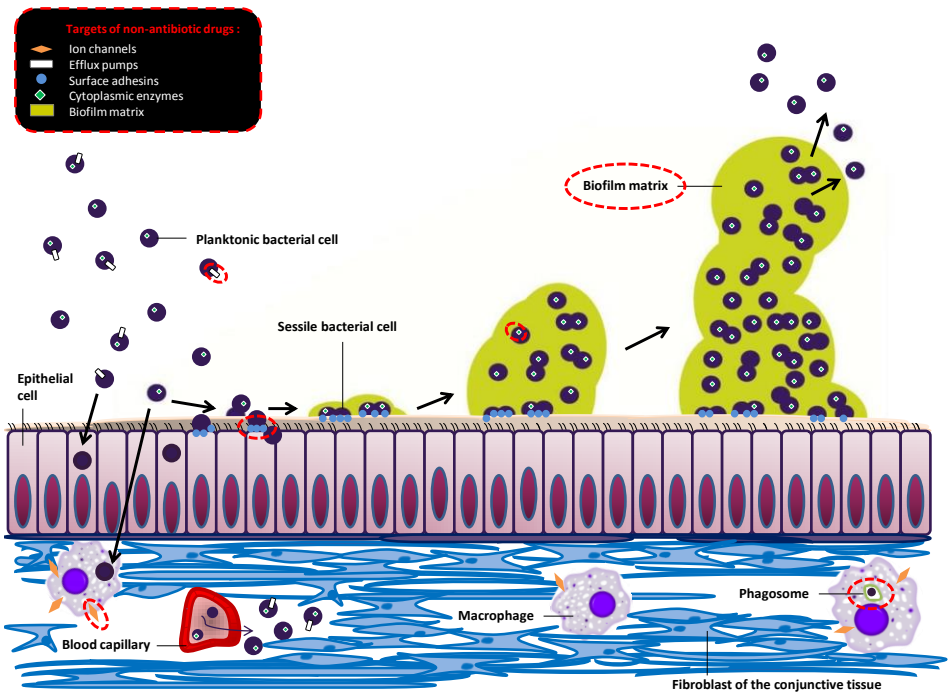
At the end of this work, we have set up two *in vitro* models of pneumococcal biofilms, viability assays and collected a series of results about (i) the development of biofilms made by *S. pneumoniae*, (ii) the activity of several antibiotics classes towards pneumococcal sessile cells and, (iii) how non-antibiotic drug, especially bronchodilators, enhance these activities through modulations of the cohesion of the biofilm matrix. Experiments and findings were respectively thought and analysed according to the clinical context of acute exacerbations of chronic bronchitis in order to reflect the clinical reality as much as possible. To this aim, an epidemiological focused on the pneumococcal resistance in Belgium was conducted in collaborations with five Belgian hospitals.

Additionally and as conclusion to this work, we have reviewed *in vitro*, *in vivo* and clinical data, available in the current literature and describing modulations, by non-antibiotic drugs, of the antibiotic activity towards respiratory bacterial pathogens. Indeed, COPD patients present frequent comorbidities, in addition to their respiratory disease and infectious episodes, and are therefore highly polymedicated. In that context, we thought that it would be interesting to summarize all the currently known beneficial or deleterious effects that non-antimicrobial drugs may have on the activity of antibiotics towards planktonic, sessile or intracellular bacterial, resistance mechanisms and life modes involved in the recurrence of infections. This work was made in the form of a paper review.



AUTHORS' CONTRIBUTIONS:

1. Conceived and designed the review: NMV, FVB;

2. Analyzed the content: NMV;

3. Wrote the paper: NMV, PMT, FVB.

Modulation of antibiotic activity towards respiratory bacterial pathogens by co-medications: review of *in vitro*, *in vivo* and clinical studies.

N.M. Vandevelde, P.M. Tulkens, F. Van Bambeke

Abstract

Discovery of novel antibiotics is slowing down. Adjuvant therapies appear thus as an appealing strategy to cope with the problem of resistance or of persistence related to specific bacterial lifestyles. Non-antibiotic drugs can modulate bacterial physiology and/or antibiotic activity. Focusing on respiratory pathogens and considering *in vitro*, *in vivo*, and clinical data, this review examines the effect of these drugs on the expression of resistance mechanisms like active efflux, the formation of biofilm and intracellular survival as well as their influence on the activity of antibiotics on bacteria growing in planktonic cultures, in biofilms, or intracellularly. Numerous beneficial effects are observed *in vitro* and often at supratherapeutic concentrations. Yet, bronchodilators improve antibiotic activity against biofilms by modifying matrix properties at concentrations mimicking those reached in the respiratory tract. Likewise, antihypertensive calcium blockers, antipsychotic phenothiazines and statins enhance intracellular killing or phagocytosis at clinically-achievable concentrations. The two last classes proved synergistic with antibiotics in clinical trials. Non-antibiotic drugs may thus offer an opportunity to increase the efficacy of antibiotics. Further studies are needed to evidence the pharmacophores responsible for their beneficial effects. It will then be possible to generate more powerful anti-infective adjuvants, exempt from their initial pharmacological action, when not useful for the treatment of the infection.

Introduction

Misuse and over-prescription of antibiotics constitute a major health problem, by contributing to the emergence or selection of bacterial resistance (Goossens *et al.*, 2005; Coenen *et al.*, 2007; van der Velden *et al.*, 2012). Moreover, therapeutic options become limited to act upon these multiresistant organisms, because the number of novel molecules coming on the market has been alarmingly decreasing over the last decade. Yet, several studies have demonstrated that non-antibiotic drugs may also harbor antibacterial properties towards a large diversity of bacterial species via modes of action that are unrelated to their main pharmacological activity. In a nutshell, these molecules may inhibit bacterial resistance mechanisms (like efflux), affect specific life modes (biofilm or intracellular persistence), and modulate tissue colonization or infection recurrence (Yang *et al.*, 2009). These drugs can also directly inhibit several proteins essential for the pathogen metabolism but different from those targeted by antibiotics. By these ways, they can present synergistic effects with antibiotics or be themselves bactericidal. The knowledge of their antimicrobial properties may therefore help improving patients' management with probably only a minimal risk of selecting resistance.

In this review, we focused our attention on the modulation, by non-antibiotic drugs, of antibiotic activity towards bacterial species causing respiratory infections. These infections account indeed for 75% of antibiotic prescriptions (Gonzales *et al.*, 2001), among which many are however unjustified (Lee *et al.*, 2014). Moreover, some of these infections can be recurrent or persistent (Dasaraju and Liu, 1996), requiring repeated administrations of antibiotics, and favoring thereby the risk of emergence of bacterial resistance. Concentrating thus on Gram-positive (namely *Streptococcus pneumoniae*, *Streptococcus mutans*, *Streptococcus. pyogenes*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Mycobacterium tuberculosis*), or Gram-negative (namely *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Chlamydia pneumoniae* and *Escherichia coli*) bacterial species, we illustrate here the different mechanisms by which non-antibiotic drugs may affect antibiotic activity *in vitro*, *in vivo* or in the clinics.

Inhibition of bacterial efflux pumps.

Efflux pumps are ubiquitous transmembrane protein transporters present in all prokaryotic and eukaryotic cells. They assure physiological and protective roles by extruding out of the cells poorly diffusible toxic compounds either produced by the endogenous cellular

metabolism or used as chemotherapeutic agents. Antibiotics represent typical toxic substances substrates for efflux transporters in bacteria (Putman *et al.*, 2000; Van Bambeke *et al.*, 2003).

According to their phylogeny, their source of energy, the number of their transmembrane spanning regions and their substrate specificity, bacterial efflux pumps belong to five superfamilies: the resistance-nodulation-division (RND) family, the major facilitator superfamily (MFS), the ATP (adenosine triphosphate)-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (Sun *et al.*, 2014). Their overexpression can increase bacterial survival during antibiotic treatment. By reducing antibiotic concentration within the bacteria, active efflux can not only bring Minimal Inhibitory Concentrations (MICs) to values higher than the clinical breakpoint (Van Bambeke *et al.*, 2003), but also select for mutations in genes coding for antibiotic targets (as commonly observed for fluoroquinolones, which leads to high-level resistance (Sun *et al.*, 2014).

In vitro, the expression of efflux pumps is often inducible after exposure to subinhibitory concentrations of their own antibiotic substrates (Avrain *et al.*, 2007; El Garch *et al.*, 2010; Chancey *et al.*, 2011). Likewise, some non-antibiotic drugs such as salicylates have been shown to induce efflux pump expression in Gram-positive bacteria like *S. aureus* by downregulating their repressor (Riordan *et al.*, 2007). Conversely, several non-antibiotic drugs have shown their capacity to act as inhibitors of efflux pumps *in vitro* (Table 1; see also [Van Bambeke *et al.*, 2010] for a review on the main classes of efflux pump inhibitors, including those deriving from non-antibiotic drugs).

Thus, the antihypertensive calcium channel blocker verapamil inhibits the efflux of fluoroquinolones and of bedaquiline and clofazime in *S. pneumoniae* and *M. tuberculosis* planktonic cultures, respectively, causing a reduction of their MICs (Pletz *et al.*, 2013; Gupta *et al.*, 2014). Of note, a synergic activity with reduced lung colonization has already been observed *in vivo* for verapamil when combined with antibiotics towards *M. tuberculosis* infections (Gupta *et al.*, 2013). In pneumococci, the inhibition of the MSF efflux pump PmrA was also associated with a reduction of mutations in the quinolone-resistance determining region (QRDR) of *gyrA*, *gyrB* and *parE* coding for the the fluoroquinolone target enzymes DNA gyrase (*gyrA*/*gyrB*) and topoisomerase IV (*parC*/*parE*), especially for efflux-positive isolates (Pletz *et al.*, 2013).

The other efflux inhibitors described all belong to drugs acting on the central nervous system. Among them, phenothiazines (e.g. chlorpromazine, thioridazine, prochlorperazine) and

thioxanthenes (e.g. trans-chlorprothixene and flupentixol) antipsychotics, tricyclic antidepressants (amitryptiline) and selective serotonin re-uptake inhibitors (SSRI) antidepressants (fluoxetine and paroxetine) are inhibiting efflux transporters present in different bacterial species (*S. aureus*, *S. pyogenes*, *P. aeruginosa* and *K. pneumoniae*) and show synergistic activity when combined with antibiotics. The mechanisms responsible for this inhibition seem to be multifactorial. It has been suggested for example that the ability of these drugs to inhibit the MFS transporters NorA responsible for fluoroquinolone resistance in *S. aureus* may rely on a direct interaction with the pump as well as on a reduction of transmembrane potential (Kaatz *et al.*, 2003b).

Noteworthy, most of these molecules were also described as inhibitors of efflux pumps in eucaryotic cells, even though transporters expressed by eucaryotic cells belong to other phylogenic families (Van Bambeke *et al.*, 2010; Marquez and Van Bambeke, 2011). This suggests possible common features associated with inhibitor recognition between procaryotic and eucaryotic transporters. It may also limit the use of these molecules *in vivo*, because of the risk of adverse effects associated with an unspecific inhibition of efflux. Moreover, in most of cases, inhibitory concentrations are far above those used in therapeutics (see Table 1), preventing from using these drugs as adjuvants of antibiotic treatment. Yet, further work may lead to the discovery of structural analogs in which pharmacological activity and inhibitory potency could be dissociated and obtained at lower, clinically-achievable concentrations.

Anti-biofilm effects

Biofilms are three-dimensional communities of sessile microorganisms adhering to a surface or interface and embedded in a matrix called extracellular polymeric substance (EPS), most often hydrated and containing polysaccharides, proteins, extracellular DNA and signaling molecules (Moscoso *et al.*, 2006; Vlastarakos *et al.*, 2007; Moscoso *et al.*, 2009). Biofilm formation is a multistep process, involving successively bacterial adhesion to a support (artificial implanted device or tissue), intensive matrix production, and release of bacterial cells allowing for colonization of other surfaces (Vlastarakos *et al.*, 2007). It is estimated that 60% of bacterial infections and up to 80% of chronic infections imply bacterial growth within biofilms (Moscoso *et al.*, 2009).

In biofilms, bacteria are highly resistant to drastic living conditions, host defenses and antibiotics, due to the matrix barrier protective role or to the acquisition of a dormant phenotype (Fux *et al.*, 2005; Roveta *et al.*, 2007; Hall-Stoodley and Stoodley, 2009; Lopez *et al.*, 2010; Simoes, 2011; Vandevelde *et al.*, 2014). Of interest, non-antibiotic drugs have

already been reported as offering new prophylactic or therapeutic strategies against biofilms both *in vitro* or *in vivo*. Their effects include (i) a decrease in matrix thickness, (ii) an inhibition of bacterial proteins involved in adhesion, matrix production or bacterial metabolism or (iii) an interference with the quorum sensing signaling (Table 2).

In vitro, the loop diuretic furosemide proved capable of destabilizing *P. aeruginosa* preformed biofilms (Cross *et al.*, 2007; Singh *et al.*, 2012). Likewise, the proton pump inhibitor esomeprazole decreased biomass and bacterial growth in biofilms formed by *S. aureus* and *P. aeruginosa* (Singh *et al.*, 2012). The relevance of these observations in the clinics is unclear, essentially because the concentrations reached by these drugs in the blood and the respiratory tract are much lower than those exerting effects on biofilms. More relevant seems to be the matrix dissassembly effect exerted by the muscarinic antagonist ipratropium on *S. pneumoniae* biofilms at concentrations mimicking those found in the epithelial lining fluid after administration of a single dose by inhalation. Moreover, this effect was accompanied by a marked improvement of the activity of antibiotics like the fluoroquinolone moxifloxacin or the fluoroketolide solithromycin (Vandevelde, 2014, submitted).

An intrinsic bactericidal effect has also been described for other benzimidazoles proton pump inhibitors (PPIs) towards *S. mutans in vit*ro planktonic cultures and biofilms (Nguyen *et al.*, 2005). This effect was observed in acidic environments (pH ≤ 5), suggesting that the activation of the IPP in its sulfenamide form was required to allow it forming disulfide bonds with bacterial proteins in order to inactivate them. Thus, bacterial enzymes inhibited by benzimidazoles include P-ATPases (but not F-ATPases) as well as enzymes involved in glycolysis (aldolase, 3-P-glyceraldehyde dehydrogenase and lactate dehydrogenase) (Nguyen *et al.*, 2005).

Non-antibiotic drugs are also able to prevent bacterial attachment to a support by inhibition of adhesins. This was the case for ipratropium when added in the culture medium of pneumococcal biofilms, but only at supra-therapeutic concentrations (Maestro *et al.*, 2007). Through a structural analogy with choline, this anticholinergic compound may inhibit different choline-binding proteins, namely the LytA amidase, the LytC lysozyme and the Pce phosporylcholinesterase. These enzymes, anchored to the cell surface, display a modular organization with a highly conserved choline-binding module that allows the binding of phosphorylcholine residues (Maestro *et al.*, 2007). They are involved in pneumococcal attachment to eukaryotic membranes and abiotic surfaces and therefore favor cells infections and biofilm formation (Moscoso *et al.*, 2006; Kadioglu *et al.*, 2008; Sanchez *et al.*, 2010). Their inhibition by ipratropium was accompanied by a loss of pneumococcal adherence and growth and even of viability within biofilms at very high concentrations (Maestro *et al.*, 2007).

The nonsteroidal anti-inflammatory drug (NSAID) ibuprofen at clinically-relevant concentrations (del Prado *et al.*, 2010) or the hypolipidemic agent simvastatin (Rosch *et al.*, 2010) also cause loss of pneumococcal adherence *in vitro,* but the underlying mechanisms have not been elucidated and are probably multifactorial. Simvastatin also protected eukaryotic cells from cytolysis induced by bacterial toxins, such as pneumolysin, and down-regulated PAFr (platelet activating factor receptor) expression involved in cell invasion (Rosch *et al.*, 2010). These effects were confirmed in mice, since simvastatin administration at a daily dose of 1mg/kg reduced tissue colonization and damages, and prolonged survival of infected animals (Rosch *et al.*, 2010).

In vivo studies have also revealed that the viral neuraminidase inhibitors used in the treatment of infections by *Influenza* viruses (oseltamivir and zanamivir, two sialic acid analogs), decrease nasopharynx colonization by pneumococcal biofilms (Trappetti *et al.*, 2009). The underlying mechanism was elucidated *in vitro* and is related to their mechanism of action. By cleaving sialic acid residues, the pneumococcal neuraminidase A (Nan A) induces adherence between bacteria and to epithelia, playing thereby an important role in biofilm matrix production, three-dimensional structure and cohesion (Soong *et al.*, 2006; Trappetti *et al.*, 2009; Parker *et al.*, 2009; Brittan *et al.*, 2012). Inhibition of the bacterial enzyme by sialic acid analogs results in a decrease of bacterial counts within the biofilm (Trappetti *et al.*, 2009) and of biofilm thickness (Vandevelde, 2014, submitted). Conversely, it was recently shown that the short acting β2-agonist salbutamol increases NanA activity at clinically-relevant concentrations, improving thereby antibiotic *in vitro* killing activity towards pneumococal biofilms (Vandevelde, 2014, submitted).

Moving now to *P. aeruginosa* biofilms, adherence was reduced of about 50% on polymers coated with salicylic acid, the major *in vivo* metabolite of acetylsalicylic acid (aspirin). This effect was accompanied by the inhibition of *las* quorum sensing system, a major regulator of biofilm production (Bryers *et al.*, 2006). Other studies confirm the beneficial effect of salicylic acid on pseudomonal biofilms, by demonstrating the inhibition of Pqs and LuxRI-type LasR quorum sensing systems (Yang *et al.*, 2009) or a reduction in bacterial counts within the biofilms (Al Bakri *et al.*, 2009).

Finally, the mucolytic agent N-acetylcystein decreases the synthesis of matrix polysaccharides by *K. pneumoniae*, reduces bacterial adherence and modifies biofilm texture *in vitro* (Olofsson *et al.*, 2003).

Effects on intracellular bacteria

A large variety of bacterial species are capable of infecting and surviving within eucaryotic cells. Among respiratory pathogens, these include *Chlamydia pneumoniae*, *Legionella pneumophila*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Van Bambeke *et al.*, 2006; Buyck *et al.*, 2013). This strategy allows them to escape immune defenses and antibiotics, and when specifically adapted to the intracellular medium, to proliferate using the eukaryotic cellular machinery. Thus, intracellular survival constitutes a pathogenic reservoir and is implicated in infection recurrence and dissemination (Bonazzi and Cossart, 2006).

Several *in vitro* and *in vivo* studies (see Table 3) have shown that non-antibiotic drugs are able to modulate bacterial survival within eukaryotic cells, macrophages in most of cases. Four main mechanisms have been proposed, namely (i) an inhibition of bacterial adherence to host cells and invasion, (ii) an inhibition of antibiotic efflux out of the macrophages enhancing their cellular accumulation (iii) a direct bactericidal activity of drugs acidifying the lysosomes, and (iv) alterations of the host cell micro-environment.

Considering bacterial adherence, experimental derivatives of hypoglycemiant biguanides can impair infection of HeLa cells by *P. aeruginosa* and *S. aureus*, by reducing the expression of β-actin, a cytoskeletal protein essential for bacterial adherence and internalization in eukaryotic cells (Olar *et al.*, 2010). Within the same category of antidiabetic drugs, derivatives of sulfonylureas rather showed direct antibacterial effects against intracellular drug-resistant *M. tuberculosis* in a model of activated human THP-1 macrophages (Wang *et al.*, 2012).

Among drugs interacting eucaryotic efflux pumps, the cholesterol-lowering drug gemfibrozil and the antihypertensive calcium channel blocker verapamil are well known as inhibitors of multidrug resistance proteins (MRP) and of P-glycoprotein, respectively. Accordingly, they have been shown to increase the intracellular activity of antibiotics substrates for these efflux transporters, namely fluoroquinolones (for MRP) and macrolides or daptomycin (for P-glycoprotein), in models of macrophages infected by *L. monocytogenes or S. aureus* (Seral *et al.*, 2003; Lemaire *et al.*, 2007).

Moving now to drugs acidifying macrophage lysosomal pH, verapamil increases the concentration of Ca^{2+} and K^{+} within the cells and stimulates the activity of Ca^{2+}-dependent V-ATPases. This effect activates hydrolytic enzymes and results in killing of intracellular bacteria, as shown for *M. tuberculosis* (Martins *et al.*, 2008). The antipsychotic

phenothiazine thioridazine also shows antituberculosis effects both *in vitro* and *in vivo* (Martins *et al.*, 2008; van Soolingen *et al.*, 2010) and increases intracellular killing of *S. aureus* as well (Ordway *et al.*, 2002; Martins *et al.*, 2004). In addition to the mechanism proposed to explain the effect of verapamil, phenothiazines also accumulate to high level in phagolysosomes, in which they could exert direct antibacterial effect (envelope lysis and intercalation within bacterial DNA [Ordway *et al.*, 2002; Martins *et al.*, 2004]). Another study suggests that phenothiazines can also inhibit *Mycobacterium* respiration (Amaral *et al.*, 1996).

Among other cell constituants, iron content is also important for bacterial proliferation. In this context, nifedipine, another calcium channel blocker, decreases the intra-macrophage iron content *in vitro* and *in vivo,* causing a slowdown of *Salmonella typhimurium* and *Chlamydia pneumoniae* proliferation within host cells (Mair *et al.*, 2011).

Of note, the effects of antipsychotic drugs and of nifedipine were observed *in vitro* at concentrations that are of the same order of magnitude than those reached in the serum of patients, opening promising perspectives for further and additional *in vivo* studies.

Activation of immune system cells

Non-antibiotic drugs can also modulate host response to bacterial infection by activating phagocytic cells killing capacities. This property has been widely described for statins (Table 4). By inhibiting HMG-CoA reductase, these cholesterol-lowering drugs enhance the formation of extracellular traps by neutrophils and macrophages both *in vitro* and *in vivo* (Chow *et al.*, 2010). These consist in networks made of extracellular fibers, primarily composed of DNA, which are produced by phagocytes and can bind pathogens and kill them through the activity of antimicrobial granule proteins such as elastase or histones. Enhanced trap formation leads to a protective effect against pneumonia induced in mice with lesser bacterial counts and inflammation in lung tissue (Chow *et al.*, 2010). Other beneficial effects of statins have been demonstrated *in vitro* and seem related to direct effects on bacteria. In Staphylocicci, simvastatin, fluvastatin and atorvastatin inhibit the bacterial HMG-CoA reductase as well as isoprene biosynthesis, leading to bacterial death (Jerwood and Cohen, 2008; Masadeh *et al.*, 2012). In pneumococci, statin bactericidal effect has not been attributed to enzymatic inhibition but rather to their hydrophobic character that enables them to perturb and degrade bacterial membranes (Bergman *et al.*, 2011). The protective effect of statins was also described in *in* vivo murine models of pneumonia caused by *S. aureus* and *S. pneumoniae.* Lower animal lethality was attributed to their combined antibacterial and anti-inflammatory properties, as demonstrated by a reduction in (i) lung and systemic bacterial

colonization, (ii) lung histopathological damages, (iii) expression of inflammatory mediators such as TNF-α, IL-1 or IL-6, and (iv) neutrophil infiltration (McDowell *et al.*, 2011; Boyd *et al.*, 2012). Of interest, these effects were observed at doses relevant of those found in plasma of patients treated for hypercholesterolemia (McDowell *et al.*, 2011). Moreover clinical data (Table 5) also show that the rate of mortality associated with community-acquired pneumonia is lower in patients receiving statins than in individuals who do not (Mortensen *et al.*, 2005), but these data are controversial (Majumdar *et al.*, 2006). A contrario, a recent clinical meta-analysis did not evidence any correlation between statin intake and reduction of mortality in sepsis (Pasin *et al.*, 2013).

Intrinsic antibacterial effect and synergy with antibiotics

In addition to specific mechanisms by which non-antibiotic drugs exert antibacterial effects, some drugs are described in the literature as possessing an intrinsic toxic effect for bacteria or showing synergism with antibiotics (Table 6).

Lacidipine, an antihypertensive dihydropyridine, proved bactericidal against planktonic cultures of different bacterial species (*S. aureus, E. coli, K. pneumoniae and P. aeruginosa*). A single dose of 30 or 60 µg decreased tissue colonization and improved survival in mice infected by *S. typhimurium* (Dasgupta *et al.*, 2007). Two of its analogs, nifedipine and amlodipine, were synergic *in vitro* with antibiotics from different classes against *E. coli* and *S. aureus* respectively (Gunics *et al.*, 2002; Kumar *et al.*, 2003; Asok *et al.*, 2004). Amlodipine also showed a synergistic effect with streptomycin towards *S. typhimurium* in mice, reducing bacterial load in the blood, the liver, and the spleen (Asok *et al.*, 2004).

Among antipsychotic drugs for which we already discussed specific antibacterial effects, synergy was observed *in vitro* for promethazine and chlomipramine with ampicillin, tetracycline or erythromycin against *E. coli* (Gunics *et al.*, 2000). More interestingly, a retrospective clinical study pointed to the efficacy of a combination of thioridazine with moxifloxacin and linezolid for the treatment of multidrug-resistant infections by *M. tuberculosis* (Table 5; Abbate *et al.*, 2012).

Lastly, promising *in vitro* data were obtained for pyrrolobenzodiazepine dimers against Gram-positive organisms, including methicillin-resistant *S. aureus* strains (MRSA) and *S. pyogenes* (Hadjivassileva *et al.*, 2005). These compounds covalently bind to DNA purines and induce interstrand cross-linking and stabilization of the double DNA helix form (Hadjivassileva *et al.*, 2007). The subsequent inhibition of DNA strands separation during replication can block the

activity of DNA polymerase and lead to antibiotic activity (Hadjivassileva *et al.*, 2007). These molecules may thus represent an opportunity for developing novel antibiotics.

Antagonism with antibiotic activity

As opposed to the beneficial effect described before, a decrease in antibiotic activity can also be observed in combination with other drugs (Table 7). Intriguingly, most of the drugs showing deleterious effects belong to the same classes or are even the same molecules as those demonstrating favorable effects, probably pointing to the importance of the model used.

In some cases, no mechanism has been proposed to explain this antagonism. This is the case for verapamil, which, at very high concentrations, reduces *E. coli* susceptibility to ampicillin in planktonic cultures (Gunics *et al.*, 2000). In other cases, modulation of host cell defense mechanisms and induction of efflux-mediated resistance have been demonstrated.

The mucolytic agent N-acetylcystein is well known for its antioxidant properties. It is therefore not surprising that it can reduce the amount of reactive oxygen species (ROS) produced by macrophages, which in its turn can impair the bactericidal effects of antibiotics. Accordingly, a decrease in intracellular potency has been observed for gentamicin and moxifloxacin when combined with N-acetylcystein against intracellular *S. aureus* (Garcia *et al.*, 2012). In the same line, N-acetylcystein has also been shown to reduce the activity of aminoglycosides and fluoroquinolones against *P. aeruginosa, K. pneumoniae and E. coli* in broth (Goswami and Jawali, 2010).

As opposed to gemfibrozil or verapamil that act as inhibitors of efflux, salicylate has been described as a inducer of this mechanism of resistance in *S. aureus,* acting by down-regulating the expression of *mgrA,* a negative regulator of the genes coding for the efflux pumps NorA, NorB, NorC, and Tet38 (Riordan *et al.*, 2007). This leads to staphylococcal resistance to ciprofloxacin (Riordan *et al.*, 2007). Other transporters could be induced as well (Price *et al.*, 2002). In addition, salicylate also represses the expression of *sarR*, a repressor of the expression of *sarA* which is required for the expression of intrinsic antimicrobial resistance in *S. aureus* (Riordan *et al.*, 2007; Rechtin *et al.*, 1999). Similarly, salicylate also induces a multiple antibiotic resistance phenotype in *K. pneumoniae* and *E. coli* (Tavio *et al.*, 2004), as well as in *M. tuberculosis* (Schaller *et al.*, 2002), characterized by an increased extrusion of norfloxacin and of antituberculosis agents, respectively. Of note, these deleterious effects were observed for salicylate concentrations relevant to those found in the

serum of patients treated by acetylsalicylic acid. Among other drugs inducing efflux, the antipsychotic haloperidol and the benzodiazepine diazepam reduce susceptibility to fluoroquinolones in *K. pneumoniae* and *E. coli* (Tavio *et al.*, 2004; Tavio *et al.*, 2012).

Finally, a very recent *in vivo* study has demonstrated that diazepam is also able to bind to receptors (α1-γ2) $GABA_{A,}$ present at the macrophages and monocytes surface, which down-regulates macrophages antibacterial activity (Sanders *et al.*, 2013). Of note, this effect seems to be specific of diazepam subunit because other benzodiazepines do not bind to the α1-$GABA_A$ subunit of the receptor (Sanders *et al.*, 2013).

Discussion

The present review has analyzed current literature data documenting the positive or negative effects of non-antibiotic drugs on the development of infections by bacteria thriving in the respiratory tract or on the activity of antibiotics.

In vitro, drug concentrations causing modulatory effects were in most cases much higher than serum levels obtained in patients receiving these medications for their registered indications. This is not surprising if taking into account that the anti-infective effects observed are generally unrelated to the main mode of action of these drugs and therefore constitute collateral effects. Although tuning down the potential clinical interest of these observations, the data generated may pave the way to structure-activity relationship studies leading to de discovery of molecules showing more potent anti-infective activity while at the same time losing their original pharmacological action.

In some specific cases, however, anti-infective effects of non-antibiotic drugs were observed at concentrations which could be achieved in humans. This was the case for antihypertensive calcium channel blockers, phenothiazine antipsychotics, statins, nonsteroidal anti-inflammatory drugs and bronchodilators. This means that they could help resolving the infection in patients for whom they are indicated. Of particular interest in this respect is the effect of bronchodilators on pneumococcal biofilms, as these drugs are currently recommended for the treatment of infectious exacerbations in COPD patients. In contrast, the benefits of nonsteroidal anti-inflammatory drugs, which are also often administered to infected patients as fever relievers, remain controversial. Indeed, at clinically-achievable concentrations, these drugs can prevent biofilm formation *in vitro* (Bryers *et al.*, 2006; Al Bakri *et al.*, 2009; Yang *et al.*, 2009; del Prado *et al.*, 2010) while at the same time inducing antibiotic efflux (Tavio *et al.*, 2004; Riordan *et al.*, 2007). Similarly, high

concentrations of the mucolytic agent N-acetylcystein often used in respiratory tract infections, rather cause detrimental effects on antibiotic activity. Noteworthy also and in contrast to what was observed for molecules active only at supratherapeutic concentrations, antibacterial effects appear here to be related to structural similarities between the eukaryotic target of these drugs and their procaryotic analogs, at least in some specific cases. This is illustrated by statins and calcium channel blockers, which interact with bacterial HMG-CoA reductase and modulate Ca^{2+} concentration within bacteria respectively (Martins *et al.*, 2008; Jerwood and Cohen, 2008; Chow *et al.*, 2010; Masadeh *et al.*, 2012). Interestingly also, antipsychotic phenothiazinic compounds shows similar effects on intracellular killing as calcium channel blockers (Martins *et al.*, 2008; van Soolingen *et al.*, 2010). This has been attributed to structural homologies between the two drug classes, more specifically the presence of aromatic rings (Asok *et al.*, 2004; Pluta *et al.*, 2011; Takacs *et al.*, 2011; Amaral and Molnar, 2012).

Conclusion

Antibiotic treatment failure remains one of the major current threats for human health. Several resistance mechanisms (including active efflux) or life modes (biofilm or intracellular persistence) reduce bacterial susceptibility to antimicrobials and therefore, favor the recurrence of infections. This review illustrated that some non-antibiotic drugs, either alone or combined with antibiotics, may be useful in this context by inhibiting bacterial resistance mechanisms or acting on persistent forms of infections. Among those molecules, calcium channels antagonists, phenothiazine compounds, statins and bronchodilators appear the most promising but mainly based on in vitro studies. Further investigations are therefore needed to better substantiate their benefit in animal models and possibly also in humans.

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Table1. Bacterial efflux pumps inhibition

Drug classes	Modulatory molecules	Human plasma levels	Modulatory doses/concentrations	Study type	Bacterial species	Main results	References
antihypertensive calcium channel blocker (phenylalkylamine)	verapamil	0.05-0.2 mg/L[a]	50 mg/L	*In vitro*	*S. pneumoniae*	Inhibition of efflux pump PmrA → ↑ intrabacterial ciprofloxacin accumulation →↓ resistance + PmrA mutation rate (>50%)	Pletz *et al.*, 2013
			50 mg/L	*In vitro*	*M. tuberculosis*	Inhibition of bedaquiline and clofazimine efflux → ↑ antibacterial activity	Gupta *et al.*, 2014
			9.4 mg/kg	*In vivo*	*M. tuberculosis*	Synergy with antibiotics → ↓ lung colonization	Gupta *et al.*, 2013
antipsychotics (phenothiazines and thioxanthenes)	chlorpromazine (1), trans-chlorprothixene (2)	0.05-0.3 mg/L[a] (1) 0.35-0.5 mg/L[b] (2) 0.5-1 mg/L[c] (3) 0.3 mg/L[d] (4) 0.002 mg/L[e] (5)	3-300 mg/L	*In vitro*	*S. aureus* *S. pneumoniae* *P. aeruginosa* *K. pneumoniae ,…*	Synergy with many antibiotics through efflux inhibition	Kristiansen *et al.*, 2010
	chlorpromazine (1), thioridazine (3)		8-12 mg/L	*In vitro*	*S. aureus* (a) *S. pyogenes* (b)	Inhibition of efflux pumps → synergy with oxacillin (a) and erythromycin (b)	Kristiansen *et al.*, 2007
	thioridazine (3), prochlorperazine (4), flupentixol (5)		4-22 mg/L	*In vitro*	*S. aureus*	Direct inhibition of efflux pump NorA + inhibition of the transmembrane electrical potential →↓ proton motive force → inhibition of MFS efflux pumps (*e.g.* NorA)	Kaatz *et al.*, 2003b
tricyclic antidepressant	amitryptiline	0.1-0.25 mg/L[a]	25-100 mg/L	*In vitro*	*S. aureus* *P. aeruginosa* *K. pneumoniae,…*	Synergy with cefuroxime, ampicillin, penicillin and tobramycin through efflux inhibition	Kristiansen *et al.*, 2010
selective serotonin re-uptake inhibitor antidepressants	paroxetine(1), femoxetine	0.002-0.02 mg/L[b]	3-30 mg/L	*In vitro*	*S. aureus*	Direct inhibition of efflux pump NorA → synergy with norfloxacin	Kaatz *et al.*, 2003a
proton pump inhibitors (PPIs) (benzimidazoles)	omeprazole	0.7 mg/L[f]	100 mg/L	*In vitro*	*S. aureus*	Direct inhibition of efflux pump NorA → synergy with norfloxacin and ciprofloxacin	Van Bambeke et al., 2010
References:	[a]Drug Information Handbook, 2010 ; [b] Bagli *et al.*, 1996 ; [C]Applied Pharmacokinetics and Pharmacodynamics - Principles of Therapeutic Drug Monitoring, 2006; [d] Dhandapani Nagasamy Venkatesh, 2009; [e] Jorgensen *et al.*, 1982 ; [f] Vlase *et al.*, 2010						
Abbreviations:	MFS : Major facilitator superfamily						
Symbols:	↓ : reduces ; ↑: increases ; → : leads to						

Table 2. Anti-biofilm effects

Drug classes	Modulatory molecules	Human plasma levels	Modulatory doses	Study type	Bacterial species	Main results	References
antihypertensive loop diuretic	furosemide	0.6 mg/L[a]	10,000 mg/L	*In vitro*	*P. aeruginosa*	↓ biofilm thickness but no effect on planktonic cells	Cross *et al.*, 2007
proton pump inhibitors (PPIs) (benzimidazoles)	esomeprazole	1.3-2.3 mg/L[b]	86 mg/L	*In vitro*	*S. aureus* (a) *P. aeruginosa* (b)	Bactericidal effect + ↓biofilm thickness and ↑ vancomycin (a) and meropenem (b) killing activities	Singh *et al.*, 2012
	lansoprazole (1), omeprazole(2)	1 mg/L[c] (1) 0.7 mg/L[d] (2)	9-185 mg/L	*In vitro*	*S. mutans*	At pH ≤5 (active form protonated), (i) inhibition of P-ATPases through the formation of disulfure bridges → ↓ bacterial acid tolerance and (ii) inhibition of the bacterial aldolase, 3-P-glyceraldéhyde dehydrogenase and lactate dehydrogenase → ↓glycolysis → bactericidal effect on planktonic and sessile cells.	Nguyen *et al.*, 2005
statins (hypolipidemic drug)	simvastatin	0.01-0.034 mg/L[e]	0.42 mg/L	*In vitro*	*S. pneumoniae*	↓ pneumococcal adherence to endothelial cells, effect of cytolytic cytokines and PAFr expression involved in cell invasion	Rosch *et al.*, 2010
			1 mg/kg	*In vivo*	*S. pneumoniae*	↓ pneumococcal adherence and PAFr expression → ↓ tissue colonization and damages + anti-inflammatory properties → ↓ mice death.	Rosch *et al.*, 2010
neuraminidase inhibitor antiviral drug	zanamivir, oseltamivir (2)	0.054-1.3 mg/L[f] (2)	10-250 mg/L	*In vitro*	*S. pneumoniae*	Neuraminidase A inhibition → ↓ bacterial counts within biofilms	Trappetti *et al.*, 2009
			1 mg/mouse	*In vivo*	*S. pneumoniae*	↓ nasopharynx biofilm colonization	Trappetti *et al.*, 2009
	zanamivir	NA	250 mg/L	*In vitro*	*S. pneumoniae*	Neuraminidase A inhibition → ↓ biofilm thickness	Vandevelde, 2014, submitted
β2-agonist bronchodilator	salbutamol	NA	7.25 mg/L	*In vitro*	*S. pneumoniae*	Neuraminidase A activation → ↓ matrix cohesion → ↑ bactericidal effect on sessile cells	
anticholinergic bronchodilator	ipratropium	NA	822-8220 mg/L	*In vitro*	*S. pneumoniae*	Choline binding proteins inhibition → ↓ pneumococcal adherence, growth (822mg/L) and viability (8220mg/L)	Maestro *et al.*, 2007
			1.45 mg/L	*In vitro*	*S. pneumoniae*	Induces an almost complete biofilm disassembly → ↑ antibiotic activity	Vandevelde, 2014, submitted
nonsteroidal anti-inflammatory drugs (NSAIDs)	ibuprofen	10-200 mg/L[g]	128 mg/L	*In vitro*	*S. pneumoniae*	↓ pneumococcal adherence and thus biofilm formation	del Prado *et al.*, 2010
	salicylic acid	100-400 mg/L (aspirin) [g]	8 mg/L	*In vitro*	*P. aeruginosa, E. coli,…*	↓ bacterial counts in planktonic cultures and within biofilms	Al Bakri *et al.*, 2009

			300 mg/L	*In vitro*	*P. aeruginosa*	Material coating with salicylate-based polymers ↓ bacterial adherence and inhibit the *las* quorum sensing system → ↓ biofilm formation	Bryers *et al.*, 2006
			1,600 mg/L	*In vitro*	*P. aeruginosa*	Inhibition of quorum sensing Pqs and LuxRI-type systems LasR and RhlRI → ↓ pyoverdine and biofilm production	Yang *et al.*, 2009
mucolytic drug	N-acetylcystein	NA	250-500 mg/L	*In vitro*	*K. pneumoniae* and other bacterial species	↓ matrix polysaccharides synthesis, and planktonic and sessile cells adherence + modification of biofilm texture → ↓ biofilm production	Olofsson *et al.*, 2003
References:	[a]Chong *et al.*, 2014 ; [b]Ullah *et al.*, 2010 ; [c]Song *et al.*, 2009 ; [d]Vlase *et al.*, 2010 ; [e]Butterfield *et al.*, 2011 ; [f] Mulla *et al.*, 2013 ; [g]Drug Information Handbook, 2010						
Abbreviations:	NA : not applicable (only concentrations in lower airways are relevant in these cases) ; PAFr : Platelet-activating factor receptor ; Pqs : Pseudomonas quinolone signal						
Symbols:	↓ : reduces ; ↑: increases ; → : leads to						

Table 3. Intracellular antibacterial effects

Drug classes	Modulatory molecules	Human plasma levels	Modulatory doses/concentrations	Study type	Bacterial species	Main results	References
biguanide (antihyperglycemic drug)	different derivates under development	ND	128-1024 mg/L	*In vitro*	*S. aureus* *P. aeruginosa*	Modulation of β-actine expression → ↓ bacterial adherence and internalization in Hela cells	Olar *et al.*, 2010
sulfonylureas	derivates under development	ND	50 mg/L	*In vitro*	*M. tuberculosis*	Bactericidal effect within THP-1 macrophages	Wang *et al.*, 2012
fibrates	gemfibrozil	15-25 mg/L[a]	62 mg/L	*In vitro*	*L. monocytogenes*	Inhibition of macrophage J774 MRPs → ↑ intracellular ciprofloxacin accumulation and killing activity	Seral *et al.*, 2003
antihypertensive calcium channel blockers	verapamil (phenylalkylamine)	0.05-0.2 mg/L[b]	9.1 mg/L	*In vitro*	*S. aureus* *L. monocytogenes*	Inhibition of macrophage J774 Pgps → ↑ intracellular azithromycin accumulation and killing activity	Seral *et al.*, 2003
			45.5 mg/L	*In vitro*	*S. aureus*	Inhibition of Pgps in THP-1 macrophage and MDCK epithelial cells → ↑ intracellular daptomycin potency but no increase in maximal efficacy	Lemaire *et al.*, 2007
			80 mg/L	*In vitro*	*M. tuberculosis*	Inhibition of Ca^{2+} and K^+ extrusion → ↑ Ca^{2+} and K^+ accumulation in macrophage → activation of the V-ATPase system → massive H^+ entry and macrophage acidosis → activation of hydrolytic enzymes → bactericidal effect	Martins *et al.*, 2008
	nifedipine (dihydropyridine)	0.10-0.13 mg/L[c]	0.09-34.6 mg/L	*In vitro*	*S. typhimurium* *C. pneumoniae*	↓ intra-macrophage iron availability → ↓ iron acquisition by intracellular bacteria → ↓ growth and proliferation	Mair *et al.*, 2011
			5 mg/kg	*In vivo*	*S. typhimurium* *C. pneumoniae*	↓serum and splenic iron amounts → ↓liver and spleen colonization and mice death	Mair *et al.*, 2011
antipsychotics (phenothiazines)	thioridazine (1) and derivates	0.5-1 mg/L[d] (1) 0.05-0.3 mg/L[b] (2)	0.1 mg/L	*In vitro*	*M. tuberculosis*	Phenothiazines accumulation in macrophages → cell acidosis and bacterial killing (through the same mechanism as for verapamil ; Martins *et al.*, 2008)	Martins *et al.*, 2008
	thioridazine		32 mg/kg	*In vivo*	*M. tuberculosis*	Thioridazine concentrates in tissues and in the intracellular compartment → impairments of the bacterial membrane integrity and interferences with Ca^{2+} transport → bactericidal effect + ↓ pulmonary tissue colonization	van Soolingen *et al.*, 2010
	thioridazine (1), chlorpromazine (2)		4-32 mg/L	*In vitro*	*M. tuberculosis*	Phenothiazines accumulation in macrophages → inhibition of bacterial respiration → bactericidal effect	Amaral *et al.*, 1996
	thioridazine (1) and chlorpromazine (2)		0.1 mg/L	*In vitro*	*S. aureus*	Phenothiazines accumulate in macrophages' lysosome → alteration and blebbing of phagocytosed *S. aureus* cell wall → bactericidal effect	Martins *et al.*, 2004
	chlorpromazine (2)		0.1 mg/L	*In vitro*	*S. aureus*	Chlorpromazine concentrates in macrophages' lysosome → bactericidal effect occurring when the lysosome fuses with the phagosome in which bacteria are internalized	Ordway *et al.*, 2002

References : [a]Forland *et al.*, 1990 ; [b]Drug Information Handbook, 2010 ; [c]Kleinbloesem *et al.*, 1984 ; [d]Applied Pharmacokinetics and Pharmacodynamics - Principles of Therapeutic Drug Monitoring, 2006

Abbreviations: ND : not determined (no current clinical use: compounds under development) ; Pgps : P-glycoproteins ; MRPs : Multidrug resistance proteins

Symbols: ↓ : reduces ; ↑: increases ; → : leads to

Table 4. Activation of immune system cells

Drug classes	Modulatory molecules	Human plasma levels	Modulatory doses/concentrations	Study type	Bacterial species	Main results	References
statins	mevastatin (1), lovastatin (2), fluvastatin (3) simvastatin (4)	0.007-0.011 mg/L[a] (1) 0.01-0.02 mg /L[b] (2) 0.45 mg/L[b] (3) 0.01-0.03 mg/L[b] (4) 0.03-0.07 mg/L[b] (5)	4-20 mg/L	*In vitro*	*S. aureus, S. pneumoniae, S. agalactiae, S. typhimurium*	↑ neutrophiles and macrophages DNA-based extracellular traps formation, dependent on bacterial HMG-CoA reductase Inhibition → bactericidal effect	Chow *et al.*, 2010
	simvastatin (4)		500 mg/kg	*In vivo*	*S. aureus*	↑ neutrophiles and macrophages DNA-based extracellular traps formation, dependent on bacterial HMG-CoA reductase Inhibition → bactericidal effect + ↓ tissue colonization	Chow *et al.*, 2010
			0.25 mg/kg	*In vivo*	*S. aureus*	↓ tissue colonization + anti-inflammatory effect → ↑ mice survival	McDowell *et al.*, 2011
			120 mg/kg = 10mg/kg/day	*In vivo*	*S. pneumoniae*	↓ tissue colonization + anti-inflammatory effect → ↑ mice survival	Boyd *et al.*, 2012
	simvastatin (4), atorvastatin (5)		29-500 mg/L	*In vitro*	*S. aureus (MRSA, MSSA)*	Inhibition of the bacterial HMG-CoA reductase → ↓ isoprenes synthesis → antibacterial activity at 29-75 mg/L (4) and 200-500 mg/L (3)	Jerwood and Cohen, 2008
			15-74 mg/L	*In vitro*	*S. aureus (MRSA, MSSA), S. epidermidis,…*	Inhibition of the bacterial HMG-CoA reductase → ↓ isoprenes synthesis → antibacterial activity at 30-74 mg/L (4) and 15-63 mg/L (5)	Masadeh *et al.*, 2012
	simvastatin (4)		15 mg/L	*In vitro*	*S. pneumoniae M. catarrhalis*	Bactericidal effect, independently on bacterial HMG-CoA reductase inhibition	Bergman *et al.*, 2011

References: [a] Ziviani *et al.*, 2001; [b]Butterfield *et al.*, 2011

Abbreviations: HMG-CoA : 3-hydroxy-3-methylglutaryl-coenzyme A

Symbols: ↓ : reduces ; ↑: increases ; → : leads to

Table 5. Antibacterial effects reported in clinical studies

Drug classes	Modulatory molecules	Human plasma levels	Modulatory doses/concentrations	Study type	Infections	Main results	References
statins	unspecified	/	unspecified	Retrospective clinical study	Community-acquired pneumonia	Statin intake ↓ infection-associated mortality and pleural effusion, independently on the activity of co-administered antibiotics	Mortensen *et al.*, 2005
	unspecified	/		Prospective cohort clinical study	Pneumonia	Statin intake ↓ bacteremia, independently on statins anti-inflammatory properties and activity of co-administered antibiotics	Majumdar *et al.*, 2006
	simvastatin (1), atorvastatin (2)	0.01-0.03 mg/L [a] (1) 0.03-0.07 mg/L [a] (2)		Meta-analysis clinical study	Sepsis	No relationship between statin intake and sepsis-related mortality	Pasin *et al.*, 2013
antipsychotics (phenothiazines)	thioridazine	0.5-1 mg/L [b]	Initial daily dose : 25 mg/day for 2 weeks Thereafter, doses increased by 25mg weekly until 200 mg/day	Retrospective clinical study	*M. tuberculosis*	Synergism with linezolid and moxifloxacin	Abbate *et al.*, 2012
References:	[a] Butterfield *et al.*, 2011 ; [b] Applied Pharmacokinetics and Pharmacodynamics - Principles of Therapeutic Drug Monitoring, 2006						
Symbols:	↓ : reduces						

Table 6. Intrinsic antibacterial effect and synergy with antibiotics

Drug classes	Modulatory molecules	Human plasma levels	Modulatory doses/concentrations	Study type	Pathogen(s)	Main results	References
antihypertensive calcium channel blocker	lacidipine (dihydropyridine)	0.002-0.02 mg/L[a]	10-200 mg/L	*In vitro*	*S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*	Bactericidal effect	Dasgupta *et al.*, 2007
			30-60 µg/mouse	*In vivo*	*S. typhimurium*	↓ tissue colonization and mice death	Dasgupta *et al.*, 2007
	nifedipine (dihydropyridine)	0.10-0.13 mg/L[b]	5 mg/L	*In vitro*	*E. coli*	Synergy with antibiotics	Gunics *et al.*, 2002
	amlodipine (dihydropyridine)	0.003-0.005 mg/L[c]	10-200 mg/L	*In vitro*	*S. aureus*	Bactericidal effect	Kumar *et al.*, 2003
			6.25-200 mg/L	*In vitro*	*S. aureus*	Through their chloride and benzene tricylic substituents, phenothiazines exert antibacterial effects and ↑ streptomycin activity	Asok *et al.*, 2004
			0.06 mg/mouse	*In vivo*	*S. typhimurium*	Synergy with streptomycin → ↓ blood, liver and spleen colonization	Asok *et al.*, 2004
antipsychotics (phenothiazines)	promethazine(1), chlomipramine (2)	0.01-0.02 mg/L[e] (1) 0.033-0.063mg/L[f] (2)	8-32 mg/L	*In vitro*	*E. coli* *S. epidermidis*	Synergy with antibiotics	Gunics *et al.*, 2000
benzodiazepines	pyrrolobenzodiazepine dimers under development	ND	0.025-0.455 µM	*In vitro*	*S. aureus*	Covalent binding to bacterial DNA → alterations of strands separation and DNA polymerase activity → inhibition of DNA replication → bactericidal effect	Hadjivassileva *et al.*, 2007
References:	[a] Da Ros *et al.*, 2003 ; [b] Kleinbloesem *et al.*, 1984 ; [c] Park *et al.*, 2006 ; [d] Drug Information Handbook, 2010 ; [e] Strenkoski-Nix *et al.*, 2000 ; [f] Herrera *et al.*, 2000						
Abbreviations :	ND : not determined (no current clinical use: compounds under development)						
Symbols:	↓ : reduces ; ↑: increases ; → : leads to						

Table 7. Antagonism with antibiotics activity

Drug classes	Modulatory molecules	Human plasma levels	Modulatory doses/concentrations	Study type	Pathogen(s)	Main results	References
antihypertensive calcium channel blocker	verapamil (phenylalkylamine)	0.05-0.2 mg/L[a]	1250 mg/L	*In vitro*	*E. coli*	↓ ampicillin activity towards planktonic cultures	Gunics *et al.*, 2000
mucolytic drug	N-acetylcystein	NA	1.6 mg/L	*In vitro*	*P. aeruginosa, K. pneumoniae, E. coli*	↓ activity of many antibiotics towards planktonic cultures	Goswami and Jawali, 2010
			4 mg/L	*In vitro*	*S.aureus*	N-acetylcystein antioxidant effect ↓ the production of reactive oxygen species related to gentamycin and moxifloxacin toxic effect towards intracellular bacteria → ↓ antibiotic killing activity	Garcia *et al.*, 2012
non-steroidal anti-inflammatory drugs (NSAIDs)	salicylate	100-400 mg/L (aspirin)[a]	320 mg/L	*In vitro*	*S. aureus*	Downregulation of efflux pump gene repressor *mgrA* and *sarR*, the repressor of gene sarA important for intrinsic bacterial resistance, decreasing bacterial susceptibility to ciprofloxacin and fusidic acid	Riordan *et al.*, 2007
			800-3200 mg/L	*In vitro*	*K. pneumoniae, E. coli*	Induction of Mar phenotype (efflux induction + porines loss) → ↓ intrabacterial antibiotic concentration → resistance	Tavio *et al.*, 2004
			80 mg/L	*In vitro*	*M. tuberculosis*	↓ killing activity of many antibiotics towards planktonic cultures	Schaller *et al.*, 2002
			320 mg/L	*In vitro*	*S. aureus*	↓ ciprofloxacin and fusidic acid activities towards planktonic cultures, independently on NorA activity	Price *et al.*, 2002
benzodiazepines	diazepam	0.2-1.5mg/L[a]	34-142 mg/L	*In vitro*	*K. pneumoniae, E. coli*	Induction of Mar phenotype (efflux induction and loss of porins) → ↓ intrabacterial antibiotic concentration → resistance	Tavio *et al.*, 2004
			71.2 mg/L	*In vitro*	*E. coli*	Induction of a MDR phenotype → induction of fluoroquinolones efflux → resistance	Tavio *et al.*, 2012
			2 mg/kg	*In vivo*	*S. aureus*	Perturbation of the down-regulation of transmission $GABA_A$ → macrophages acidosis → ↓ cytokine production, phagocytosis and killing of bacteria. Of note, benzodiazepines that are not targeting the α1-$GABA_A$ subunit do not affect macrophages function	Sanders *et al.*, 2013
antipsychotics (phenothiazines)	haloperidol	0.005-0.02 mg/L[a]	150 mg/L	*In vitro*	*E. coli*	Induction of a MDR phenotype → induction of fluoroquinolones efflux → resistance	Tavio *et al.*, 2012
References:	[a] Drug Information Handbook, 2010						
Abbreviations :	NA : not applicable (only concentrations in lower airways are relevant in these cases ; MDR : Multi-drug resistance						
Symbols:	↓ : reduces ; → : leads to						

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