Universidade do Minho
Escola de Engenharia

Tânia Raquel Rodrigues Grainha

Insights into interspecies interactions, phenotypic profile and antimicrobial susceptibility in an artificial ventilator-associated pneumonia microbiome

October 2016

Insights into interspecies interactions, phenotypic profile and antimicrobial susceptibility in an artificial ventilator-associated pneumonia microbiome

Tânia Raquel Rodrigues Grainha

UMinho | 2016

Universidade do Minho
Escola de Engenharia

Tânia Raquel Rodrigues Grainha

Insights into interspecies interactions, phenotypic profile and antimicrobial susceptibility in an artificial ventilator-associated pneumonia microbiome

Master Dissertation

Master in Bioengineering

Work Supervised by:

Professor Maria Olívia Pereira

And Co-supervised by:

Doctor Susana Patrícia Lopes

Doctor Maria Elisa Rodrigues

October 2016

DECLARAÇÃO

Nome: Tânia Raquel Rodrigues Grainha

Endereço eletrónico: raquel.r-1993@hotmail.com

Título da tese: Insights into interspecies interactions, phenotypic profile and antimicrobial susceptibility in an artificial ventilator-associated pneumonia microbiome

Orientadora: Professora Maria Olívia Pereira

Co-orientadoras: Doutora Susana Patrícia Lopes

Doutora Maria Elisa Rodrigues

Ano de conclusão: 2016

Mestrado em Bioengenharia

Universidade do Minho, Outubro de 2016

ACKNOWLEDGMENTS/AGRADECIMENTOS

Terminada esta importante jornada, não posso deixar de reconhecer que a realização desta dissertação teve de forma direta ou indireta, o contributo fundamental de muitas pessoas.

Em primeiro lugar queria agradecer às minhas orientadoras, Professora Maria Olívia Pereira, Doutora Susana Lopes e Doutora Elisa Rodrigues pela orientação, pelos conhecimentos transmitidos e por todas as sugestões essenciais para a elaboração do trabalho.

Não posso deixar de agradecer também a todo o grupo MOP, em particular à Diana e à Paula, pela cooperação e disponibilidade demonstradas e pelos conhecimentos transmitidos ao longo do ano.

À Vanessa porque teve sempre presente, pelas nossas brincadeiras e pela amizade. Às colegas de laboratório (Vanessa, Carla, Sílvia), pela boa disposição e companheirismo.

Gostaria de agradecer à Ângela França pelas úteis sugestões e contributos na extração e análise do RNA e ao Luís Melo pelo seu imprescindível contributo na citometria de fluxo, pela disponibilidade e aprendizagem transmitida.

Não poderia deixar de fazer um agradecimento especial à Andreia que foi uma ajuda importante na realização deste trabalho, pela amizade, apoio e motivação nos momentos mais complicados.

Por fim agradeço à minha família e ao meu namorado pelo apoio e carinho.

This work was supported by ESCMID Research Grants 2014 and the Portuguese Foundation for Science and Technology (FCT), under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684), by FCT and the European Community fund FEDER, through Program COMPETE, under the scope of project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462).

ABSTRACT

Ventilator associated pneumonia (VAP) is the second most common nosocomial infection in the intensive care units (ICU) and the most common in mechanically ventilated patients. VAP presents a serious problem in ICU due to high mortality and morbidity rates associated, because it is often biofilm-mediated and polymicrobial. Therefore, understanding the impact of microorganisms in VAP and their interaction is a major challenge posed. Additionally, the ineffective current treatment strategies have led to the emergence of new approaches to fight these polymicrobial consortia, with a great number intervening in the quorum-sensing (QS) intercellular communication.

This work aimed to give insights into the behavior of bacterial-fungal communities involving *Pseudomonas aeruginosa* and *Candida albicans* associated to VAP, when exposed to different antimicrobial approaches. For this, single- and mixed-species biofilms were thoroughly characterized in terms of cultivable cells and biomass after 24 h treatment with conventional drugs (amphotericin B, AmB; polymyxin B, PolyB) and alternative agents, in particular QS inhibitors (QSI) from different sources (commercial drugs: salicylic acid, ciprofloxacin (CIP), azithromycin (AZT); natural sources: chlorogenic acid, farnesol, linalool, patulin) and enzymes (alginate lyase, desoxirribonuclease), tested alone or in combination.

Results showed that the combination AmB+PolyB did not affect the pre-established *P. aeruginosa* and *C. albicans* consortia. Interestingly, excepting for patulin, QSI agents were effective at reducing biofilm-encased cells, in particular single-species biofilms. CIP showed a great potential to inhibit both single-and mixed-species biofilms. Linalool was also effective in disturbing *C. albicans* in single and mixed biofilms. Contrariwise, enzymes had no effect against biofilms. Regarding double combinations, the addition of farnesol or linalool to CIP led to similar results from that obtained with CIP alone, with reductions observed in biofilm-encased cells. In general, the addition of a third agent - particularly in the case of chlorogenic acid - did not significantly improve the effect of AmB+PolyB or farnesol/linalool+CIP combinations.

Additionally, efforts were made to characterize the un- and treated dual-species biofilms by flow cytometry and RNA sequencing (RNA-seq), however no accurate results were obtained due to unexpected methodological hitches.

In conclusion, the use of new approaches seems to be a promise in treating bacterial-fungal consortia often involved in VAP. This work showed that combining different agents from distinct sources is a valuable option to control *P. aeruginosa* and *C. albicans* biofilms. Nevertheless, optimization on the antimicrobial doses and further clinical studies are urgently required to improve therapy effectiveness and avoid additional costs.

RESUMO

A pneumonia associada à ventilação (PAV) é a segunda infeção nosocomial mais comum em unidades de cuidados intensivos (UCI) e a mais comum em pacientes sob ventilação mecânica. A PAV apresenta um problema grave na UCI devido às elevadas taxas de mortalidade e morbilidade associadas, porque muitas vezes é mediada por biofilmes e tem caráter polimicrobiano. Compreender o impacto dos microrganismos em PAV e suas interações é um grande desafio que se coloca. Além disso, a ineficiência das estratégias de tratamento atuais levaram ao surgimento de novas abordagens para combater esses consórcios polimicrobianos, com um grande número intervindo na comunicação intercelular quorum-sensing (QS).

Este trabalho teve como objetivo o conhecimento do comportamento das comunidades bacterianas-fúngicas envolvendo *Pseudomonas aeruginosa* e *Candida albicans* associadas a PAV, quando expostas a diferentes estratégias antimicrobianas. Para isso, os biofilmes simples e mistos foram caracterizados em termos de células cultiváveis e biomassa após 24 h de tratamento com medicamentos convencionais (anfotericina B, AmB; polimixina B, PolyB) e com agentes alternativos, em particular, inibidores de QS (QSI) obtidos de diferentes fontes (medicamentos comerciais: ácido salicílico, ciprofloxacina (CIP), azitromicina (AZT); fontes naturais: ácido clorogénico, farnesol, linalol, patulina); enzimas (alginato liase, desoxirribonuclease), testados isoladamente ou em combinação.

Os resultados mostraram que a combinação AmB+PolyB não afetou os consórcios pré-estabelecidos de *P. aeruginosa* e *C. albicans*. Curiosamente, com exceção de patulina, os agentes IQS foram eficazes na redução de células de biofilme, em particular nos biofilmes formados por uma única espécie. A CIP mostrou grande potencial para inibir biofilmes simples e mistos. O linalol também foi eficaz contra *C. albicans* em biofilmes simples e mistos. Pelo contrário, as enzimas não tiveram efeito contra os biofilmes. No que se refere às combinações duplas, a adição de farnesol ou linalol à CIP conduziu a resultados semelhantes obtidos apenas com CIP, com reduções observadas em células envolto-biofilme. Em geral, a adição de um terceiro agente - em particular no caso do ácido clorogénico - não melhorou significativamente o efeito das combinações AmB+PolyB ou farnesol/linalol+CIP.

Adicionalmente, foram feitos esforços para caraterizar os biofilmes de dupla espécie, não tratados e tratados, por citometria de fluxo e sequenciação de RNA (RNA-seq), contudo não foram obtidos resultados fiáveis devido a dificuldades metodológicas inesperadas.

Em conclusão, o uso de novas estratégias parece ser uma promessa no tratamento de consórcios bacterianos-fúngicos frequentemente envolvidas em PAV. Este trabalho mostrou que a combinação de diferentes agentes obtidos a partir de fontes distintas é uma opção valiosa para controlar biofilmes de *P. aeruginosa* e *C. albicans*. No entanto, é necessário a otimização das doses de antimicrobianos e mais estudos clínicos para melhorar a eficácia da terapia e evitar custos adicionais.

AIMS AND OUTLINE OF THE THESIS

This study proposed to look into the behavior of inter-kingdom biofilms, as well as to find new approaches to treat these polymicrobial infections. In this regard, it was intended to characterize the communities involving a bacterial species, *P. aeruginosa*, and a fungal species, *C. albicans*, often found in VAP, as well as its susceptibility towards different treatment strategies involving combinations of antimicrobials with QS inhibitors (QSI). Further, a transcriptomic analysis of the un- and treated dual-species biofilms was attempted.

This thesis is organized into three chapters. Chapter 1 briefly reviews relevant clinical aspects of VAP, emphasizing the composition of microbial communities involved in VAP. The microbial biofilms, including their relevant particularities and their importance for VAP are also summarized in this chapter.

In Chapter 2, the microorganisms, culture conditions, materials and techniques used in the work presented herein are described.

Chapter 3 reports studies on mono- and dual-species biofilms of *Pseudomonas aeruginosa* and *Candida albicans*, often associated to VAP. These studies include microbial compositions of mono- and dual-species populations, before and after antimicrobial exposure.

Chapter 4 includes the main conclusions of the work displayed and proposes future research lines finalizing the thesis.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND ACRONYMS

°C: Celsius degrees
%: Percent
µL: microliter
AI: Auto inducer
ANOVA: Analysis of variance
AmB: Amphotericin B
AZT: Azithromycin
CIP: Ciprofloxacin
CFU: Colony-forming units
CV: Crystal violet
DNA: Deoxyribonucleic acid
cDNA: complementary DNA
eDNA: extracelular DNA
***e.g.*:** (*exempli gratia*) for example
EPS: Extracellular polymeric substances
***et al.*:** (*et alli*) and others
ETT: Endotracheal tube
***g*:** Relative centrifugal force
h: hour
ICU: Intensive Care Units
kHz: kilo-Hertz
L: Liter
log_{10}: logarithm with base 10
m/s: meter per secound
MBC: Minimum inhibitory concentration
MBEC: Minimum biofilm eradication concentration
MBIC: Minimum biofilm inhibitory concentration
MDR: Multidrug resistant
mg: milligram
MIC: Minimum Inhibitory Concentration
min: minute
mL: milliliter
M: Molar
MRSA: Methicillin resistant *Staphylococcus aureus*
MSSA: Methicillin sensitive *Staphylococcus aureus*
NaCl: Sodium chloride
OD: Optical density
OD570 nm: Optical density at 570 nm
OD640 nm: Optical density at 640 nm
P: probability
PBS: Phosphate-buffered saline
PCL: Periciliary liquid layer
pH: potential hydrogen
PI: Propidium iodide

PNA: Peptide nucleic acid
PolyB: Polymyxin B
PQS: Pseudomonas quinolone signal
PS: Polystyrene
Psl: Polysaccharide synthesis locus
Q2: Rate of epithelial oxygen consumption
QS: Quorum-sensing
QSI: Quorum-sensing inhibitors
rpm: revolutions per minute
RNA: Ribosomal Ribonucleic Acid
RNA-seq: Ribosomal Ribonucleic Acid sequencing
RPMI: Roswell Park Memorial Institute
RQI: RNA quality indicator
s: Second
SD: Standard deviation
SDA: Sabouraud dextrose agar
SDB: Sabouraud dextrose broth
TSA: Tryptic soy agar
TSB: Tryptic soy broth
TTSS: Type three secretion system
UP: ultrapure
VAP: Ventilator-associated pneumonia
VF: Virulence factors
V: volume
V/V: volume-to-volume
W/V: weight-to-volume

SCIENTIFIC OUTPUT

Abstracts in conferences

Grainha, Tânia; Rodrigues, M. Elisa; Lopes, Susana P.; Lourenço, Anália; Henriques, Mariana; Pereira, Maria Olívia; Insights into *Pseudomonas aeruginosa* and *Candida albicans* consortia challenged by antimicrobials. Biofilms 7 - Microbial Works of Art. No. P3: 76, Porto, Portugal, June 26-28, 2016.

ISBN: 978-989-97478-7-6

Chapter 1

GENERAL INTRODUCTION

1.1 Ventilator-associated pneumonia: a general overview of the disease

Mechanical ventilation can be understood as ventilatory assistance and can be seen as the maintenance of the oxygenation and/or ventilation of patients in an artificial manner [1]. In general, mechanical ventilation is crucial when clinical signs indicate that the patient cannot maintain an airway or adequate oxygenation or ventilation [2]. Clinical situations when assisted ventilation is crucial to include: exacerbation of chronic obstructive pulmonary disease (COPD), cardiogenic pulmonary edema, acute hypoxemic respiratory failure and in some immunosuppressed patients with pulmonary infiltrates and respiratory failure [3, 4].

Mechanical ventilation can be noninvasive, involving various types of face masks, or invasive, involving endotracheal intubation [2]. Whenever possible, the patient is only subjected to noninvasive ventilation; however, there are cases where it is necessary to reccur to invasive therapy. In the latter case, a plastic tube is usually inserted through the nose or mouth into the trache. If mechanical ventilation is needed for more than a few days, the tube may be inserted directly into the trachea through a small incision in the front of the neck, in a procedure named tracheostomy. A tracheostomy is safer and more comfortable for longer periods of ventilation [5].

Mechanical ventilation is a life-saving procedure, but it is also associated with some complications, presenting risks for the patients [6]. The presence of an endotracheal tube (ETT) in invasive ventilation causes risk of sinusitis, tracheal stenosis, vocal cord injury, and, very rarely, tracheal-esophageal, tracheal-vascular fistula or ventilator associated pneumonia (VAP) [2]. The presence of the ETT is the principal risk factor for the development of VAP [7], because the existence of this tube results in a change of natural defense mechanisms against microaspiration around the tube [8]. The tube modifies the anatomy of the larynx, causing damage in the mucociliary system, and acting as an entry point for microorganisms to the lower airways, through microbial adherence and multiplication on the tracheal surface [9]. Infectious bacteria obtain direct access to the lower respiratory tract via: microaspiration, which can occur during intubation itself; development of a biofilm (typically encompassing Gram-negative bacteria and fungal species) within the ETT; pooling and trickling of secretions around the cuff; and impairment of mucociliary clearance of secretions with gravity dependence of mucus flow within the airways [10, 11]. The pathogens involved in this disease can be of endogenous or exogenous origin. The exogenous sources include aerosols of the contamineted air and medical devices (ventilatory circuit, catheter, bronchoscope and humidifier).

The endogenous sources are mostly from the oral, pharyngeal and gastric flora of the patient [12, 13].

Naturally, the defense mechanisms against lung infection include: the anatomy of airways as a first line of defense, mucus production, cought reflex, mucociliary clearance, lactoferrin, basement membrane and the immune system [12, 14].

By definition, VAP is a nosocomial infection that is developed as a consequence of intubation and mechanical ventilation, presenting serious problems in the intensive care units (ICU). It is the second most common nosocomial infection in the ICU and the most common in mechanically ventilated patients [15], presenting in some cases non-specific symptoms or clinical signs [16].

VAP contributes to approximately half of all cases of hospital-acquired pneumonia [4], being one common cause of mortality and morbidity within the ICU. It occurs after 48 hours or more of tracheal intubation, affecting 9 to 27% patients receiving mechanical ventilation [4]. Frequently, VAP may be classified as early onset or late onset, depending on the days when it occurs after the start of ventilation. Early onset pneumonia occurs within four days of intubation and mechanical ventilation and late onset pneumonia only develops after four days [8].

It is very difficult to clinically diagnose VAP infection. Nevertheless, it is suspected when certain conditions are identified in addition to the presence of a persistent pathogen on chest radiography. It is necessary to check, at least, two of the following conditions: temperature higher than 38 °C, leukocytosis (>12×10^9 white blood cells/L) or leukopenia (<4×10^9 white blood cells/L) and purulent tracheal secretions [17].

1.2 Microorganisms involved in VAP infection

A diverse range of microorganisms are often involved in VAP infection, with bacteria predominating in the VAP microbiome. Microorganisms responsible for the infection differ according to the population of patients in the ICU, the times of hospital and ICU stays, and the specific diagnostic method(s) used [18].

According to the time of infection, microorganisms colonizing the ETT can be divided into two groups. Typically, early onset VAP is caused by pathogens that are sensitive to antibiotics (e.g.: *Streptococcus* spp., *Haemophilus* spp., methicillin sensitive *Staphylococcus aureus* (MSSA), antibiotic-sensitive enteric Gram-negative bacilli, *Escherichia coli*, *Klebsiella pneumonia*,

Enterobacter spp., *Proteus* spp. and *Serratia marcescens*). Conversely, late onset VAP is caused by multidrug resistant (MDR) pathogens (e.g.: methicillin resistant *Staphylococcus aureus* (MRSA), *Acinetobacter* spp*., P. aeruginosa*), which are extremely difficult to treat upon colonization [6, 8, 11]. Whilst there is no clinical information about the cause of the infectious agent, this information is relevant to initiate an adequate antibiotic therapy, normally using broad-spectrum antibiotics [16].

Such as in many diseases, not all microorganisms cause infection with the same frequency (Figure 1).

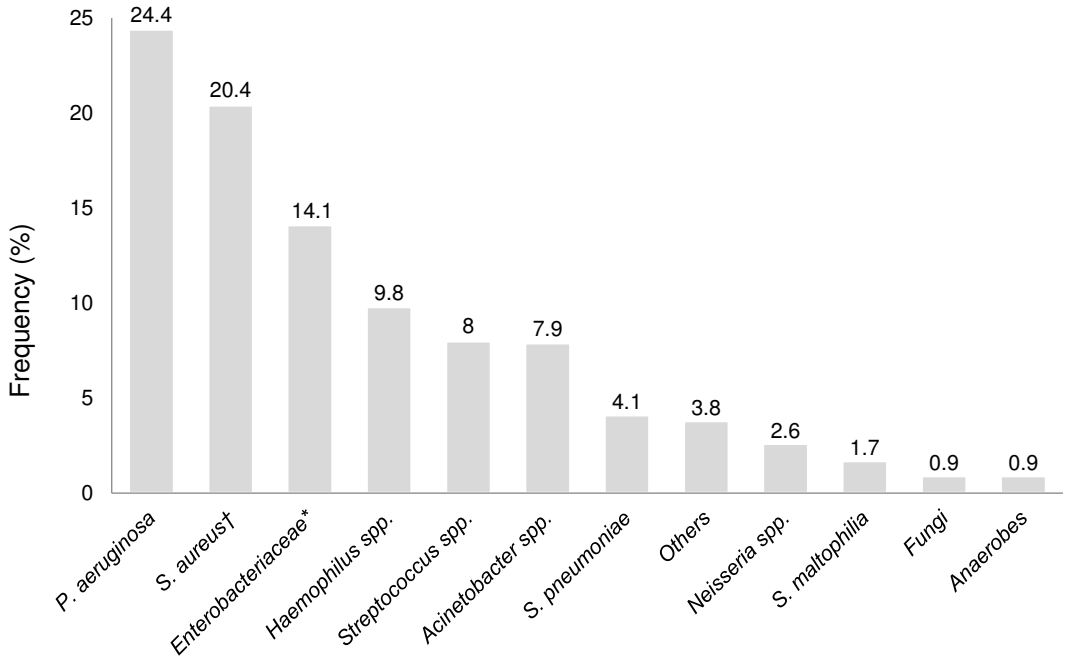


Figure 1 Microorganisms involved in VAP infection and respective frequency (based in 24 studies). †Distribution when specified: methicillin-resistant *S. aureus*, 55.7 %; methicillin-sensitive *S. aureus*, 44.3 %. *Distribution when specified: *Escherichia coli*, 24.1%; *Proteus* spp., 22.3 %; *Enterobacter* spp., 18.8 %; *Klebsiella* spp., 15.6 %; *Serratia* spp., 12.1 %; *Citrobacter* spp., 5.0 %; *Hafnia alvei*, 2.1 %. *S. maltophilia: Stenotrophomonas maltophilia.* Adapted from [18].

P. aeruginosa and *S. aureus* are both important bacterial opportunistic pathogens and are frequently isolated from catheters, including the VAP ETT, as well as skin, eyes, and respiratory tract infections [19]. One of the most prevalent pathogen causing VAP is *P. aeruginosa* [4, 18], which contributes to high morbidity and mortality rates in ICUs and makes the treatment very difficult due to its intrinsic antibiotic resistance mechanisms (e.g.: multidrug efflux pumps, the presence of an outer membrane, enzymatic drug changes and target changes). The existence of

the outer membrane is an important resistance factor of this pathogen due to existing porin channels that mediate the uptake of the antibiotic [20].

In addiction, numerous reports have also indicted the coexistence of bacterial and fungal species, namely *P. aeruginosa* and *C. albicans*, in a variety of different opportunistic infections, namely in VAP infections [21].

P. aeruginosa is a Gram-negative bacteria found in environments such as soil and water but being able to live even in hostile environments, its occurrence is common in other environments [22, 23]. This opportunistic pathogen is frequently isolated from healthy humans as part of the human microbiota and can coexist in mixed infections with *C. albicans* [24]. *C. albicans,* another opportunistic human pathogen is a polymorphic fungus that can grow either as yeasts (ovoid-shaped), as pseudohyphae and hyphae morphologies [25]. This microorganism is frequently found as part of the normal microbiota of the skin, gastrointestinal tract and female genital tract [26] and is a major cause of opportunistic infections that range from superficial infections to life-threatening systemic infections [27].

1.3 Quorum-sensing: A Sophisticated Communication System

When a microorganism interferes with other microorganism, it is called inter-cellular communication. This communication may result in the worsening of the infection (e.g.: increase of antibiotic resistance) or even bring benefits to the host (e.g.: microbial competition at the site of infection).

The situation of negative association can be said that is the 'ideal situation' as it is the most advantageous condition for the host. This means that one of the microorganisms in the surrounding environment will act to the detriment of the other, leading to a lower escalation of the disease and the patient own clinical state.

Different interactions can occur in a polymicrobial infection, including those between pathogens and between the pathogens and the host. If the severity of polymicrobial infection is equivalent to the sum of infection with each alone then it present an additive interaction. If the gravity of polymicrobial infection is greater than the sum of infection with each pathogen alone, then can be termed ‘synergism’ or if on the contrary the severity of polymicrobial infection is less than in case of infection with each alone that there is an antagonistic interaction [28].

Bacteria and fungi influence each other directly or indirectly in different ways (e.g.: use of metabolic by-products, physical interactions, chemical exchanges or changes in the environment) [28].

Several studies aim to evaluate the interaction of *P. aeruginosa* and *C. albicans.* The interaction of *C. albicans* and *P. aeruginosa* is often mediated by quorum sensing (QS) molecules produced by both microorganisms [29].

Biofilm formation by many pathogens is related to a form of inter-bacterial communication known as QS [30]. Microorganisms generally use QS to develop the biofilm by sensing the bacterial load and constructing a well-organized community with intimate relationships of competition or cooperation [31]. QS is a mechanism of cell-to-cell communication in which small diffusible signaling molecules, called autoinducers (AI), globally regulate gene expression. The bacteria sense their population density through these molecules and when a particular threshold concentration of AI is reached these signal molecules can bind and activate receptors inside bacterial cells. These receptors can alter gene expression to activate behaviors that are beneficial under the particular condition [32, 33]. Signaling by AI in the QS system forms the basis for alterations in various gene expressions including virulence factors VF, secretion system, motility, sporulation, and biofilm formation [34]. The production of VF by QS is achieved by high cell densities within a biofilm [35]. Using QS, bacterial populations can switch from acting as individual cells to operating in a concerted to a multi-cellular fashion [30].

Some examples of microbial interaction models between microorganisms frequently associated with pneumonia are summarized in Tables 1 (positive interactions) and 2 (negative interactions).

Table 1 Positive microbial interaction models between microorganisms frequently associated with pneumonia

First microorganism colonization or infection	Potential interacting pathogen	Interaction	Ref.
Bacteria			
P. aeruginosa	*S. aureus*	Selection for *S. aureus* small-colony variants by a *P. aeruginosa* exoproduct, 4-hydroxy-2-heptylquinoline-N-oxide	[19]
Bordetella pertussis	*IFV (PR8)*	Suppression of early innate host response mediated by pertussis toxin	[36]
Legionella pneumophila	*Aspergillus* sp.	Invasive aspergillosis	[37]

Table 1 (Continued) Positive microbial interaction models between microorganisms frequently associated with pneumonia

Fungi			
Candida spp.	*P. aeruginosa*	*Candida* impedes alveolar macrophage reactive oxygen species production and is correlated with an increase of *P. aeruginosa* pneumonia	[38]
C. albicans	*P. aeruginosa*	*C. albicans* ethanol stimulates *P. aeruginosa* strain PAO1 biofilm formation on airway cells	[39]
Viruses			
Adenovirus (types 1,2,3 and 5)	*S. pneumoniae*	The virus particles increase bacterial adherence to respiratory tract epithelial cells	[40]
Coronavirus NL63	*S. pneumoniae*	Increased adherence of *S. pneumoniae* to infected cells correlated with an increased expression level of the platelet-activating factor receptor	[41]
CMV	*A. actinomycetemcomitans*	Increased susceptibility for bacterial adherence to cells	[42]
IFV type A	*H. influenzae*	Increase in bacterial adherence to epithelial cells due to increased cell surface eukaryotic receptors	[43]
IFV type A	*N. meningitidis*	Enhancement in meningococcal adhesion to epithelial cells due to viral neuraminidase	[44]
IFV type A	*S. pneumoniae*	Inhibition of pneumococcal clearance due to platelet-activating factor receptor	[19]
IFV type A	*S. pyogenes*	Increase in bacterial adherence to epithelial cells by the presence of GAS (group A Streptococcus) capsule and secondary bacterial superinfection	[45]
IFV type A	Ia group B streptococci	Secondary bacterial infection by influenza	[46]
IFV type A (H1N1)	*B. parapertussis*	Impairment of bacterial clearance mediated by chemokine MIP-2	[47]
IFV type A (H1N1)	*S. aureus*	Viral hemagglutinin increase the efficiency of internalization of *S. aureus*	[48]

Table 1 (Continued) Positive microbial interaction models between microorganisms frequently associated with pneumonia

IFV type B	*S. pneumoniae*	Severe secondary pneumonia due influenza B infection	[49]
IFV type B	*S. pyogenes*	Severe secondary pneumonia due influenza B infection	[49]
Metapneumovirus	*S. pneumoniae*	Severe secondary pneumonia	[50]
PIF 4	*C. pneumoniae*	Fatal hemorrhagic pneumonia	[51]
PIF 3	*H. influenzae*	Increase in bacterial adherence to epithelial cells due to increased cell surface eukaryotic receptors	[43]
PIF 3	*S. pneumoniae*	Increase in bacterial adherence to epithelial cells due to increased cell surface eukaryotic receptors	[43]
RSV	*H. influenzae*	Bacterial adhesion to transfected cells mediated by Glycoprotein G/NTHI outer membrane P5-fimbria	[52, 53]
RSV	*P. aeruginosa*	Increase in bacterial adherence to epithelial cells by the presence of Glycoprotein G	[54]
RSV	Metapneumovirus	Severe bronchiolitis	[55]
RSV	*S. pneumoniae*	Bacterial adhesion to G‐protein-transfected cells due to the presence of this protein	[56]
Rhinovirus	*H. influenzae*	Bacterial and viral pathogens interact to cause pulmonary disease exacerbation	[57]
Rhinovirus	*S. pneumoniae*	Increased pneumococcal colonization via increases platelet-activating factor receptor	[58]
Rhinovirus	*S. aureus*	Increase in the efficiency of internalization of S. aureus by the secretion of inflammatory cytokines (IL-6 and IL-8) and overexpression of ICAM-1	[59]

Table 2 Negative microbial interaction models between microorganisms frequently associated with pneumonia

First microorganism colonization or infection	Potential interacting pathogen	Interaction	Ref.
Bacteria			
Commensal lung microbiota	IFV type A	Proper activation of inflammasomes generates virus-specific CD4 and CD8 T cells, and antibody responses	[60]
A. baumannii	*C. albicans*	*C. albicans* filamentation inhibition	[56]
H. influenzae	*M. catarrhalis*	Decreased colonization	[61]
H. influenzae	*S. pneumoniae*	Complement and neutrophils provide rapid clearance of *S. pneumoniae*	[62]
L. gasseri	*S. aureus*	Bactericidal activity of hydrogen peroxide results in *S. aureus* killing	[63]
L. acidophilus	*C. albicans*	Enhanced clearance of *C. albicans* correlated with both early mRNA gene expression for interleukin (IL)-4 and interferon (IFN)-g	[64]
L. paracasei	*S. aureus*	Bactericidal activity of hydrogen peroxide results in *S. aureus* killing	[65]
P. aeruginosa	*C. albicans*	Virulence factors of *P. aeruginosa* results in filamentous *C. albicans* killing and biofilm formation on fungal filaments	[66]
P. aeruginosa	*C. albicans*	*C. albicans* secreted proteins directly suppress *P. aeruginosa* pyoverdine and pyochelin expression	[67]
P. aeruginosa	Pathogenic fungi†	Growth inhibition of fungi	[28]
P. aeruginosa	*S. aureus*	S. aureus inhibition by antistaphylococcal substance produced by *P. aeruginosa*	[55]
P. aeruginosa	*S. epidermidis*	QS-controlled factors from *P. aeruginosa* prevents staphylococcal growth	[68]
S. enterica	*C. albicans*	*C. albicans* filamentation inhibition mediated by *sop*B effectors	[69]
S. aureus	*H. influenzae*	Decreased colonization	[61]
S. aureus	*S. pneumoniae*	Decreased colonization	[61]

Table 2 (Continued) Negative microbial interaction models between microorganisms frequently associated with pneumonia

S. warneri	*Legionella* sp.	Anti-*Legionella* peptide inhibits *Legionella* growth	[70]
S. maltophilia	Pathogenic fungi‡	Growth inhibition of fungi	[71]
S. gordonii	*S. mutans*	Bactericidal activity of hydrogen peroxide results in *S. mutants* death	[72]
S. oligofermentans	*S. mutans*	Suppression of *S. oligofermentans* growth due bactericidal activity of hydrogen peroxide	[73]
S. pneumoniae	*S. aureus*	Bactericidal activity of hydrogen peroxide results in *S. aureus* death	[74]
S. sanguinis	*S. mutans*	Bactericidal activity of hydrogen peroxide results in *S. mutants* death	[72]
Fungi			
Pneumocystis murina	IFV type A	Specific antibody response increases viral clearance	[75]

In the literature, a wide set of interaction with different organisms have been studied. Tables 1 and 2 presented previously show that positive interactions mainly occur with by viruses the first microorganism colonization or infection. On the other hand, when bacteria are the first microorganism colonization it generally leads to negative interaction with potential interacting pathogen. This study is very important in clinical setting since it allows developing therapies considering these consortia in infections.

In polymicrobial synergism infection, the combined effect of two or more microbes on the disease progression can be more dramatic that any of the individuals alone and it display enhanced pathogen persistence in the infection site, increased disease severity, and increased antimicrobial resistance [76, 77]. Synergetic interactions lead to biofilm development with increase of antibiotic tolerance, defense against competitors, adaptation to changing environments, increased tissue damage and declined pulmonary infection [76, 78].

However, in some cases the antagonistic interactions between organisms within a community are unavoidable due to competition for limited resources, with effects on the growth or viability of competitors [79].

In VAP infections these both situations can occur as have seen in the tables and in case of inter-kingdom consortia of *P. aeruginosa* and *C. albicans* these situations were also observed [29, 66, 80–82].

Understanding the mechanisms of increased pathogenicity in polymicrobial infections is the first step in the development of an effective therapy. Inter-cellular communication is a challenge which has been working in an attempt to minimize the injury from infection to the host.

1.3.1 *Pseudomonas aeruginosa* quorum-sensing mechanisms

There are numerous VF possessed *by P. aeruginosa,* which are closely related with virulence mechanisms of this pathogen. These mechanisms include QS, type II and type III secretion system (TTSS). The main VF described for *P. aeruginosa*: QS, type three secretion system (TTSS) and the presence of lipopolysaccharides.

The VF include proteases, lipases and exotoxin A released by a type II secretion system (Xcp regulon), as well as exotoxins (such as Exo S, Exo T, Exo U and Exo Y) secreted by TTSS [83]. In addiction pyoverdine, rhamnolipids, lipopolysaccharide (LPS) and pili also are included in virulence of *P. aeruginosa* [84].

The TTSS, a needle-like protein apparatus able to inject exotoxins (mentioned above) into cells is active against human macrophages, where Exo U expression represents a risk factor for mortality in *P. aeruginosa* pneumonia [85]. The production of elastase, phenazines (e.g.: pyocyanin) and TTSS exotoxins by bacteria is associated to lung injuries. For instance, elastase was associated with acute lung injury and Exo U was associated with increased virulence and a high risk of bacteremia [86]. Conversely, the presence of lipopolysaccharide, namely Lipid A, interacts with receptor 4 in epithelial cells but apparently has not an apparent effect in pneumonia [87].

Another VF common in majority of microorganisms is the capacity to form biofilm since become resistant to antimicrobials.

Many classes of AI have been described to date. The most intensely studied AI are the acylhomoserine lactones (AHLs) of Gram-negative bacteria (e.g.: *P. aeruginosa*), the oligopeptides produced by Gram-positive bacteria (e.g.: *S. aureus and S. pneumoniae*) and AI-2 used a wide range of Gram-positive and Gram-negative bacterial species, whose structures remain unknown in most cases [33].

P. aeruginosa generally uses QS to coordinate the production of VF by the two main signaling systems, Las and Rhl which are controlled by the genes namely *las* and *rhl*, respectively [86]. These systems utilize self-secreted AI molecules 3-oxo-dodecanoyl acyl homoserine lactone (3-oxo-C12-HSL, one of the AHL molecules) and N-butanoyl acyl homoserine lactone (C4-HSL), respectively. When high concentrations of these molecules are achieved it's possible dock with their cognate receptor proteins such as LasR and RhlR and form a signal-receptor complex, which regulates the expression of various genes responsible for biofilm formation and virulence factor production [88]. The systems LasI/Las and RhlI/RhlR are responsible for the secretion of elastase and rhamnolipids respectively [89]. Additionally, a third system of QS known as Pseudomonas Quinolone Signal (PQS) has been reported in this organism [90] and is involved in secretion of pyocyanin [89].

It is estimated that QS in *P. aeruginosa* regulate 350 genes, of which around 30% encode VF production [91].

1.3.2 *Candida albicans* quorum-sensing mechanisms

The VF and abilities of *C. albicans* potentiate this microorganism to infect a diverse host niches. For this pathogen, the virulence is mostly due to morphological transition between yeast and hyphal forms. Its ability to shift form yeast to filamentous forms is controlled by QS. The filamentous forms of the yeast such as hyphae or pseudo-hyphae, are responsible for tissues penetration and destroy macrophages [92]. The hyphal form has been shown to be more invasive than the yeast form [25]. On the other hand the smaller yeast form is believed to represent the form primarily involved in dissemination [93]. The yeast-to-hyphal form transition can be controlled by temperature, pH, nutrient concentration, cell density or human serum [94]. While at low pH (<6) *C. albicans* grow predominantly in the yeast form, at high pH (>7) the hyphal growth is induced. Cell density also affect the morphology due to QS, high cell densities (>10^7 cells ml^{-1}) favor yeast growth, while low cell densities (<10^7 cells ml^{-1}) promotes hyphal growth. The transition between these two morphologies is termed dimorphism and it has been proposed like an important factor of pathogenicity [95].

The attributes of this pathogen further include the expression of adhesins and invasins on the cell surface which mediate adherence to other cells (*C. albicans* cells, other microorganisms or host cells) and to abiotic surfaces. The grip may be mediated by the expression of the ALS gene family,

belonging to the family of the immunoglobulins. These agglutinins interact specifically with molecules of the host [96]. Phenotypic switching, secretion of hydrolytic enzymes, thigmotropism and formation of biofilms are included in virulence factors of this pathogen. *C. albicans* is able to form biofilms on abiotic (catheters, dentures) or biotic (mucosal cell) surfaces which shows that an important virulence factor [97]. In addition, others aspects include rapid adaptation to fluctuations in environmental pH, metabolic flexibility, powerful nutrient acquisition systems and robust stress response machineries [98].

C.albicans has the greatest number of QS molecules identified until now. The first QS molecules identified for this fungus were tryptophol and phenylethyl alcohol [99]. These molecules inhibit cell grow and germ tube formation. Additionally three molecules have been identified from *C. albicans*: farnesol, tyrosol and farnesoic acid [100], although this last one has only been reported in *C. albicans* ATCC 10231 [101].

Farnesol and tyrosol belong to sesquiterpene alcohols. These inducers regulate cell morphology, growth, biofilm formation, resistance to oxidative stress, and other processes in the life of *C. albicans*.

Farnesol is the best QS molecule studied and it is known to blocks the morphological transition from yeast to the filament form at high cell densities [102], otherwise tyrosol promotes filamentation [100].

1.3.3 *Pseudomonas aeruginosa* and *Candida albicans* interaction

In case of interaction between *P. aeruginosa* and *C. albicans,* positive or negative associations may occur. QS molecule 3-oxo-C12-HSL produced by *P. aeruginosa* affect the morphology of *C. albicans* (inhibits *C. albicans* filamentation), thereby altering the ability of the fungus adhere or to invade tissues and the capacity to form biofilm [103]. In this interaction, the surveillance of *C. albicans* is compromised and it decreases the chance of subsequent infection. Therefore, it represents a negative association between the microorganisms, but also a positive situation for the host. In addition, bacterial toxins such as pseudomonal phenazines have been shown to have antifungal properties [66, 80]. *C. albicans* can also influence negatively *P. aeruginosa* by producing farnesol, a QS molecule similar in structure to 3-oxo-C12-HSL, which at low cell density allows modulate the behavior of *P. aeruginosa* and decreases its virulence. This decrease in virulence is achieved due to inhibition of PQS production (Cugini et al. 2007) required

for the expression of several VF [104]. At higher concentration, this molecule can suppress the effect of farnesol on PqsR activity [29].

However, positive associations between these pathogens are also been reported [38]. The main molecular mechanisms of interaction between *P. aeruginosa* and *C. albicans* are outlined in Figure 4.

The understanding of the molecular details of QS mechanisms and the way they affect host cells provide an important tool that are now considered a target in antibiotic treatment and controlling bacterial infections.

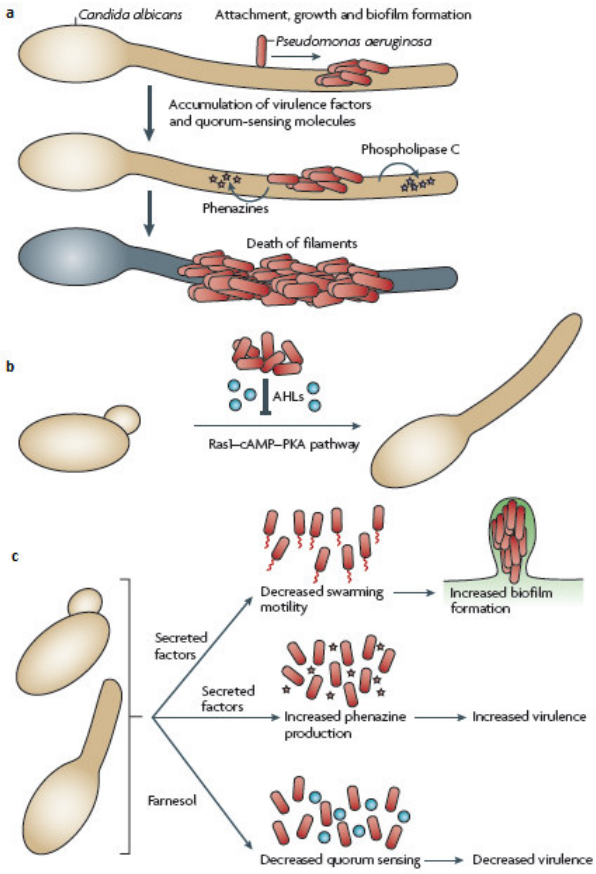


Figure 2 Molecular mechanisms of the interactions between *P. aeruginosa* and *C. albicans* [28] **a|** *P. aeruginosa* can attach to the surface of *C. albicans* hyphae and form biofilms. Production of VF (phospholipase C [66] and phenazines [80] by *P. aeruginosa* leads to the death of the fungal filament **b|** QS molecules that are produced by both *P. aeruginosa* and *C. albicans* in the mixed-species biofilm [29]. *P. aeruginosa* produces 3-oxo-C12- homoserine lactone that can inhibit the Ras1–cyclic AMP (cAMP)– protein kinase A (PKA) pathway for hyphal growth in *C. albicans*, inhibiting filamentation of the fungus [81]. Because yeast cells have increased survival in the presence of *P. aeruginosa*, the switch to growth as yeast may contribute to the coexistence of both species in mixed infections **c|** The farnesol produced by *C. albicans* allows modulates the behavior [29] of *P. aeruginosa* and decreased quorum sensing molecules. Others uncharacterized *C. albicans* factors increase the production of VF or alter swarming motility and biofilm formation [80, 82].

1.4 Biofilms in VAP

One factor that deserves high attention in VAP is the ability of the microorganisms to develop biofilms. Biofilms are well-structured microbial communities adhered to a surface, where microorganisms are enclosed by a self-produced matrix [105]. These extracellular polymeric substances (EPS) are produced when exopolysaccharides, adhesins and cognate receptors are synthesized by planktonic cells when attached to the surface, which may be epithelial cells or medical devices. The EPS matrix, which can constitute up to 90% of the biofilm biomass [53], is a complex mixture including DNA, proteins, polysaccharides and other macromolecules, conferring a protective effect to biofilm-encased microorganisms against aggressive external factors [52, 106, 107].

The initial attachment of microorganisms to the surface (Phase 1) is driven by hydrophobic and/or electrostatic interactions as well as specific bacterial surface molecules. The next step is the formation of micro- and then macro-colonies (Phase 2) together with the formation of the polymeric matrix (Phase 3). Lastly, when maturation occurs, the enlarged biofilm shows focal dissolution and begins to release planktonic bacterial cells (Phase 4), which can spread to other locations and develop other biofilms. A schematic representation of biofilm formation phases are shown in figure below.

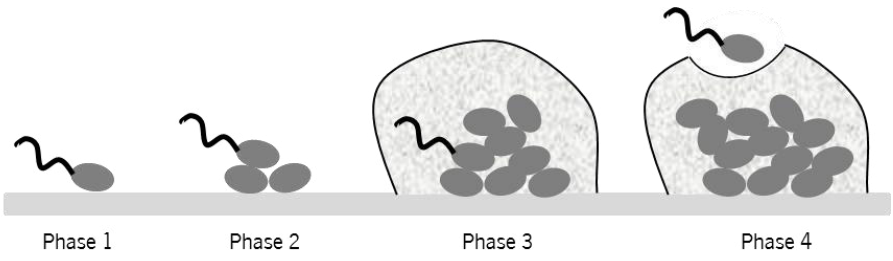


Figure 3 Stages of biofilm formation. Initial attachment of microorganisms to the surface (Phase 1); formation of micro-colonies (Phase 2); formation of macro-colonies with the formation of a self-produced polymeric matrix (Phase 3); maturation of biofilm, with the release of planktonic cells.

Biofilms are often resistant against antimicrobial agents (such as antibiotics), due to several mechanisms (e.g.: the presence of the EPS, presence of dormant cells and multicellular resistance strategies).

Biofilm structure itself acts as a barrier to host defenses, because it reduces the mobility of immune cells and restricts antibiotic diffusion, contributing for the chronicity of the disease [106, 107]. Mechanisms of biofilms resistance against antimicrobials agents include:

- Presence of EPS: A biofilm is a permanent source of infection and confers protection to the microorganisms towards antibiotic therapy due to the presence of the EPS matrix [108]. The EPS of biofilms contains polysaccharides, proteins, and DNA (eDNA) that form a glue-like substance for adhesion to the surface and for the three-dimensional biofilm architecture [109]. The EPS function as a barrier providing protection to the cells in the biofilm, and is considered one of the causes associated to antimicrobial resistance, where the antimicrobial agents may be prevented from penetrating the biofilm if they bind to components of the biofilm matrix or to microorganism membranes [110]. Alginate is an polysaccharide of EPS in a mucoid *P. aeruginosa* biofilm and has been shown to increase the tolerance to aminoglycosides [111].

- Multicellular strategies: additional to the protective matrix, biofilm resistance depends on different multicellular strategies from exchanged plasmids, transposons and mutations in genes encoding microbial resistance [107, 112]. The high densities of microorganisms within the biofilm under pressure of antimicrobials enhance horizontal gene transfer and the frequency of mutation [113]. Additionally, delay of antimicrobial penetration through EPS can also induce the expression of genes mediating resistance in the biofilm [114].

- Altered metabolism: the expression of distinct metabolic pathways based on the local environmental circumstances in the biofilm is controlled by various genotypes and phenotypes coexist within the biofilm population. Studies have shown that biofilms feature chemical patterns that correspond to gradient of antimicrobials with differences in concentration from outside to inside the biofilm. Due this fact he metabolic activity of microorganisms is higher in the external part of the biofilm and lower in the internal part leading to a reduced susceptibility to antimicrobials [110, 115] In this case the metabolism is adapted due to external factors.

- Slow growth: biofilm is a metabolic heterogeneous population induced by multiple microcolonies part of such communities. Within the biofilms are created zones characterized by an poor nutrition and can be developed stationary phase-like dormant-cells which can lead for antimicrobials resistance [110, 116] by restricting the access for these antimicrobials that contribute to the general resistance seen in biofilms [117].

- Persister cells: some cells of microorganisms can survive after prolonged exposure to antimicrobials which are denominated persister cells. This subpopulation of microorganisms that differentiate into a dormant and protected state. One aspect of chronic characteristics of bacterial infectious disease is the presence of these dormant cells, which are able to resist to the action of most antibiotics. These dormant bacteria, which colonizes specifically the deeper parts of the biofilms, can suppress their metabolism, including cell membrane formation, protein synthesis, and DNA replication [118, 119]. Dormant bacteria can survive to antibiotic exposure because their antibiotic target sites are deactivated, which means that they tolerate sublethal concentrations of antibiotics [120, 121].

- Oxygen condition: the oxygen tension in the depth of biofilm is low such as been described for *P. aeruginosa* and these low tension (hypoxia) change the composition of multidrug efflux pumps with consequently antimicrobial resistance like response to stress [122]. Aditionlly, the ability of this bacterium to adapt to the oxygen-limited environments is associated with a drastic physiological change in *P. aeruginosa* (e.g. increased alginate production; alterations in the outer membrane; biofilm development), which contributes to an increased antibiotic tolerance [123]. In addition, the anaerobic environment within biofilms will most likely affect aminoglycoside antimicrobial activity due to the downregulation of energy metabolism genes [124] and by triggering changes in gene expression [125].

- Swarming: the microorganisms with the ability to swarm reflect a social multicellular behavior and its reflets in decrease the effectiveness of the agents against infections [126].

- QS: it is complex system, which regulates the behaviour with others cells, control of microbial population density and expression of VF [127].

Biofilms are seen as a major problem in medical settings and it has been estimated that up to 80% of all infections worldwide are biofilm-related [128].

Therefore, microbial adhesion and biofilm formation on medical devices (Figure 4) often leads to deterioration, blockage and loss of function and removal of the devices is often the unique solution [129].

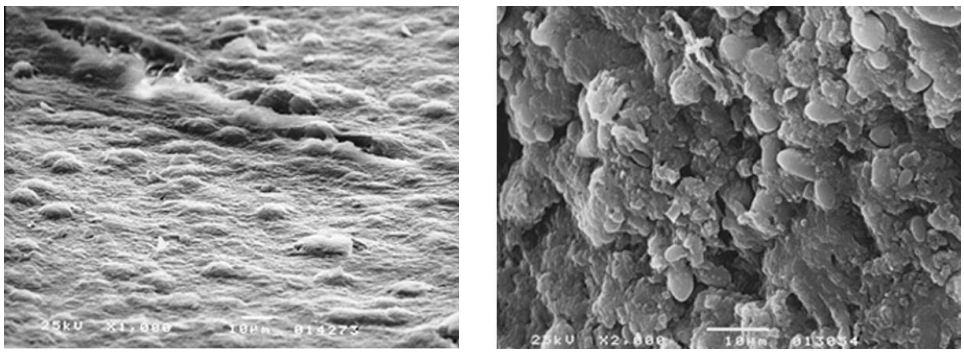


Figure 4 Photomicrographs captured by scanning electron microscopy of biofilms developed on the VAP ETT [16].

Biofilms are typically formed on the inner surface and in case of the VAP infection in ETT [130] which contribute to the development of the infection, allowing the contact and persistence of pathogens within the host [131]. In ventilated patients the biofilms forms on ETT very quickly after intubation and its act as a significant source of inoculation of the lungs by bacteria [132]. The tube appears to be a point of access of microorganisms to the lower respiratory tract (e.g.: via microaspiration during intubation itself, development of biofilm within the tube) [10, 11].

1.5 VAP therapy

VAP patients still receive inadequate initial antibiotics treatment even if it is well known that the incidence of MDR pathogen infections is on the rise in ICU. VAP is one of the major sites for emergence of MDR pathogens because subtherapeutic antibiotic concentrations in the lung require longer duration of therapy, thereby favoring selection of resistant bacteria [16].

When VAP is suspected, empirical antibiotics should be administered immediately. The accurate identification of etiologic pathogens might improve therapy procedures and the control of the infection to avoid complications for the patient. Although bacteriological sampling is important, it should not significantly delay the start of treatment [14]. Even if the bacteriological test is not available, the therapy should be initiated. The most relevant information to start treatment, until there is no specific clinical information on the cause of the infection agent, it's whether it comes to early onset VAP or late onset VAP. Usually, the antibiotic therapy is initiated with a broad-spectrum antibiotic [16]. It is still necessary to know the whole clinical history of the patient with infection to choose the appropriate treatment.

Approximately 50 % of all antibiotics administered in ICUs are for treatment of VAP [8].

The usual duration of treatment for early onset VAP is eight days and longer in the case of late-onset VAP or if MDR organisms are suspected or identified [133, 134]. Late onset VAP requires broad spectrum antibiotic whereas early onset VAP can be treated with limited spectrum antibiotics [4].

1.5.1 Conventional therapy

There is a general agreement that rapid initiation of appropriate antimicrobial therapy improves the outcome of the disease [14]. In VAP, conventional therapy typically includes the administration of empirical antibiotics in an attempt to cure the infection. However, due to certain resistance mechanisms, specific to each microorganism, the recommended treatment varies. The therapy is started according to the time of onset (Table 3).

Table 3 Comparison of recommended initial empiric therapy for VAP according to time of onset [4, 133]

Early onset VAP	**Late onset VAP**
Second or third generation cephalosporin: e. g.,	Cephalosporin
ceftriaxone: 2 g daily;	e. g., cefepime: 1–2 g every 8 hours;
cefuroxime: 1.5 g every 8 hours;	ceftazidime 2 g every 8 hours
cefotaxime: 2 g every 8 hours	OR
OR	Carbepenem
Fluoroquinolones	e. g., imipenem + cilastin: 500 mg every 6 hours or 1 g
e. g., levofloxacin: 750 mg daily;	every 8 hours;

Table 3 (Continued) Comparison of recommended initial empiric therapy for VAP according to time of onset [4, 133]

e. g., levofloxacin: 750 mg daily; moxifloxacin: 400 mg daily OR Aminopenicillin + beta-lactamase inhibitor e. g., ampicillin + sulbactam: 3 g every 8 hours OR Ertapenem 1 g daily	meropenem: 1 g every 8 hours OR Beta-lactam/beta-lactamase inhibitor e. g.: piperacillin + tazobactam: 4.5 g every 6 hours PLUS Aminoglycoside e. g.: amikacin: 20 mg/kg/day; gentamicin: 7 mg/kg/day; tobramycin: 7 mg/kg/day OR Antipseudomonal fluoroquinolone e. g.: ciprofloxacin 400 mg every 8 hours; levofloxacin 750 mg daily PLUS Coverage for MRSA e. g.: vancomycin: 15 mg/kg every 12 hours OR linezolid: 600 mg every 12 hours

The therapy can be initiated based on the information about the causative microorganism (Table 4).

Owing to the high rate of resistance to monotherapy observed with *P. aeruginosa,* combination therapy is always recommended [135].

Table 4 Recommended therapy for suspected or confirmed multidrug resistant organisms and fungal VAP [4, 133]

Pathogen	**Treatment**
Methicillin-resistant *Staphylococcus aureus*	See Table 3
Pseudomonas aeruginosa	Double coverage recommended. See Table 3

Table 4 (Continued) Recommended therapy for suspected or confirmed multidrug resistant organisms and fungal VAP [4, 133]

Acinetobacter species	Carbapenem e. g.: imipenem + cilastin; 1 g every 8 hours; meropenem 1 g every 8 hours OR Beta-Lactam/beta-lactamase inhibitor e. g., ampicillin + sulbactam: 3 g every 8 hours OR Tigecycline: 100 mg loading dose, then 50 mg every 12 hours
Extended-spectrum beta-lactamase (ESBL) positive enterobacteriaceae	Carbepenem e. g.: imipenem + cilastin: 1 g every 8 hours; meropenem: 1 g every 8 hours
Fungi	Fluconazole: 800 mg every 12 hours; caspofungin: 70 mg loading dose, then 50 mg daily; voriconazole (for aspergillus species): 4 mg/kg every 12 hours
Legionella	Macrolides (e. g.: azithromycin) OR Fluoroquinolones (e. g.: levofloxacin)

Several antibiotics have re-emerged as alternatives to treat *P. aeruginosa*. Polymyxins are used in cases of MDR *P. aeruginosa*. Polymyxin B (PolyB) is an antibiotic primarily used for resistant Gram-negative infections and frequently used to control pulmonary infections caused by *P. aeruginosa* [136]. For treatment of *C. albicans*, amphotericin B (AmB) was considered the best choice to treat serious and invasive *Candida* infections [137].

1.5.2 Alternative approaches in biofilm control: the QS inhibitors

Due to the frequent failures of antibiotherapy towards biofilms new therapeutic modalities to treat the infection are required. Additionally, empirical antimicrobial regimen leads to the overuse of antibiotics and, thus, emergence of antimicrobial resistance. This is a major problem associated with the use of antibiotics and a constant concern as it leads to increased mortality. The formation

of biofilm in the VAP ETT is also a concern, because this is usually impervious to systemic antibiotics, making the treatment of infection more complicated [106].

For *P. aeruginosa,* high percentages of isolates resistant to aminoglycosides, ceftazidime, fluoroquinolones, piperacillin/tazobactam, and carbapenems were reported from several countries in Europe in 2011 [138].

To improve the quality of care and consequent decrease in mortality associated with VAP, new challenges are placed in the field of treatment of infection. The alternatives to conventional therapies are related by using new products that may interact and interfere more effectively with microorganisms causing the disease, leading to more efficient therapy. The new strategies generally aim to interfere with exoproducts produced by microorganisms, inhibiting cellular communication and reducing biofilm production. Possible alternatives to synthetic antimicrobials include QSI, antimicrobial peptides, biofilm degradation enzymes and bacteriophages.

- Antimicrobial peptides: antimicrobial peptides (AMPs) are part of the inmate immune response found among all classes of life. Yours characteristics hydrophobic and cationic allow them pouring and fragment the citoplasmatic membrane [139]. Recently was described that certain AMPs are able to destabilize EPS by binding to the eDNA with consequent disrupt of biofilm [140].

- Enzymes: new approaches to biofilm control also include the use of biofilm matrix-degrading enzymes in order to detach the cells of biofilms and make them more susceptible to antimicrobial. DNase I is one of these enzymes which degrades the eDNA of EPS. Dispersin B (DspB) and α-amylase act likewise DNase on the biofilms. A combination of the enzymes with antibiotics is promising in clinical context [141].

- Bacteriophages: also known as phages, are viruses that infect bacteria. Thus, phages are candidates to prevent and control biofilm since they are able infect and lyse cells in single and polymicrobial species biofilms [142, 143]. However bacteria can escape to phage infection by increasing your biofilm formation ability [144].

- QS inhibitors (QSI): in this regard, advances in the QS field have been made. In recent years, a number of biotechnology companies that aim specifically at developing anti-

QS and anti-biofilm drugs have emerged [145]. The great diversity of QS signal generators, receivers, carriers, regulators and the signals themselves, represent multiple targets for inhibition of QS. This strategy of inhibition of QS pathways by interfering with signal generation, signal relay, signal transduction or destruction of the signal entirely is also known as "quorum quenching" [146]. QS inhibition strategies may have targeted species-specific QS molecules. Many studies have been done and many compounds studied with potential QSI were proposed such as furanone [147] and patulin [148], two compounds of natural origin. Some antibiotics (such as the macrolide azithromycin (AZT), the β-lactam ceftazidime and the fluoroquinolone ciprofloxacin (CIP)) have QSI activity in addition to their conventional antibiotic activity [149]. QSI are compounds able to quench the action of and usually are considered as safe. Additionally, it is not expectable any development of resistance since QSI not impose any selective pressure to bacteria, as seen in classical antibiotic treatment [150].

REFERENCES

1. **Ventilação Mecânica (1ª parte)** [http://estudmed.com.sapo.pt/anestesiologia/ventilacao_mecanica_1.htm]

2. **Overview of Mechanical Ventilation - Critical Care Medicine - Merck Manual Professional Edition** [https://www.merckmanuals.com/professional/critical-care-medicine/respiratory-failure-and-mechanical-ventilation/overview-of-mechanical-ventilation]

3. Brochard L: **Mechanical ventilation: invasive versus noninvasive**. *Eur Respir J* 2003, **22**(47 suppl).

4. **Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia.** *Am J Respir Crit Care Med* 2005, **171**:388–416.

5. **Mechanical Ventilation - Lung and Airway Disorders - Merck Manual Home Edition** [https://www.merckmanuals.com/home/lung-and-airway-disorders/respiratory-failure-and-acute-respiratory-distress-syndrome/mechanical-ventilation]

6. Pneumatikos IA, Dragoumanis CK, Bouros DE: **Ventilator-associated pneumonia or endotracheal tube-associated pneumonia? An approach to the pathogenesis and preventive strategies emphasizing the importance of endotracheal tube.** *Anesthesiology* 2009, **110**:673–80.

7. Zolfaghari PS, Wyncoll DLA: **The tracheal tube: gateway to ventilator-associated pneumonia.** *Crit Care* 2011, **15**:310.

8. Hunter JD: **Ventilator associated pneumonia**. *BMJ* 2012, **344**:e3325–e3325.

9. Pinciroli R, Mietto C, Berra L: **Respiratory therapy device modifications to prevent ventilator-associated pneumonia.** *Curr Opin Infect Dis* 2013, **26**:175–83.

10. Grgurich PE, Hudcova J, Lei Y, Sarwar A, Craven DE: **Diagnosis of ventilator-associated pneumonia: controversies and working toward a gold standard.** *Curr Opin Infect Dis* 2013, **26**:140–50.

11. Mietto C, Pinciroli R, Patel N, Berra L: **Ventilator associated pneumonia: evolving definitions and preventive strategies.** *Respir Care* 2013, **58**:990–1007.

12. Safdar N, Crnich CJ, Maki DG: **The pathogenesis of ventilator-associated pneumonia: its relevance to developing effective strategies for prevention.** *Respir Care* 2005, **50**:725–39; discussion 739–41.

13. Joseph NM, Sistla S, Dutta TK, Badhe AS, Parija SC: **Ventilator-associated pneumonia: a review.** *Eur J Intern Med* 2010, **21**:360–8.

14. Hunter JD: **Ventilator associated pneumonia.** *Postgrad Med J* 2006, **82**:172–8.

15. Afshari A, Pagani L, Harbarth S: **Year in review 2011: Critical Care - infection**. *Crit Care* 2012, **16**:242.

16. Piazza O, Wang X: **A translational approach to ventilator associated pneumonia.** *Clin Transl Med* 2014, **3**:26.

17. Kalanuria AA, Zai W, Mirski M: **Ventilator-associated pneumonia in the ICU.** *Crit Care* 2014, **18**:208.

18. Chastre J, Fagon J-Y: **Ventilator-associated pneumonia.** *Am J Respir Crit Care Med* 2002, **165**:867–903.

19. Hoffman LR, Déziel E, D'Argenio DA, Lépine F, Emerson J, McNamara S, Gibson RL, Ramsey BW, Miller SI: **Selection for Staphylococcus aureus small-colony variants due to growth in the presence of Pseudomonas aeruginosa.** *Proc Natl Acad Sci U S A* 2006, **103**:19890–5.

20. Lambert PA: **Mechanisms of antibiotic resistance in Pseudomonas aeruginosa.** *J R Soc Med* 2002, **95 Suppl 4**:22–6.

21. Nseir S, Jozefowicz E, Cavestri B, Sendid B, Di Pompeo C, Dewavrin F, Favory R, Roussel-Delvallez M, Durocher A: **Impact of antifungal treatment on Candida-Pseudomonas interaction: a preliminary retrospective case-control study.** *Intensive Care Med* 2007, **33**:137–42.

22. Pier GB: **Pulmonary disease associated with Pseudomonas aeruginosa in cystic fibrosis: current status of the host-bacterium interaction.** *J Infect Dis* 1985, **151**:575–80.

23. Tan MW, Mahajan-Miklos S, Ausubel FM: **Killing of Caenorhabditis elegans by Pseudomonas aeruginosa used to model mammalian bacterial pathogenesis.** *Proc Natl Acad Sci U S A* 1999, **96**:715–20.

24. Kaleli I, Cevahir N, Demir M, Yildirim U, Sahin R: **Anticandidal activity of Pseudomonas aeruginosa strains isolated from clinical specimens.** *Mycoses* 2007, **50**:74–8.

25. Berman J, Sudbery PE: **Candida Albicans: a molecular revolution built on lessons from budding yeast.** *Nat Rev Genet* 2002, **3**:918–30.

26. Morales DK, Hogan DA: **Candida albicans interactions with bacteria in the context of human health and disease.** *PLoS Pathog* 2010, **6**:e1000886.

27. Sandven P: **Epidemiology of candidemia.** *Rev Iberoam Micol* 2000, **17**:73–81.

28. Peleg AY, Hogan DA, Mylonakis E: **Medically important bacterial-fungal interactions.** *Nat Rev Microbiol* 2010, **8**:340–9.

29. Cugini C, Calfee MW, Farrow JM, Morales DK, Pesci EC, Hogan DA: **Farnesol, a common sesquiterpene, inhibits PQS production in Pseudomonas aeruginosa.** *Mol Microbiol* 2007, **65**:896–906.

30. Kaufmann GF, Park J, Janda KD: **Bacterial quorum sensing: a new target for anti-infective immunotherapy.** *Expert Opin Biol Ther* 2008, **8**:719–24.

31. Li Y-H, Tian X: **Quorum sensing and bacterial social interactions in biofilms.** *Sensors (Basel)* 2012, **12**:2519–38.

32. Bassler BL, Losick R: **Bacterially speaking.** *Cell* 2006, **125**:237–46.

33. Antunes LCM, Ferreira RBR: **Intercellular communication in bacteria.** *Crit Rev Microbiol* 2009, **35**:69–80.

34. Fuqua WC, Winans SC, Greenberg EP: **Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators.** *J Bacteriol* 1994, **176**:269–75.

35. Tegmark K, Morfeldt E, Arvidson S: **Regulation of agr-dependent virulence genes in Staphylococcus aureus by RNAIII from coagulase-negative staphylococci.** *J Bacteriol* 1998, **180**:3181–6.

36. Ayala VI, Teijaro JR, Farber DL, Dorsey SG, Carbonetti NH: **Bordetella pertussis infection exacerbates influenza virus infection through pertussis toxin-mediated suppression of innate immunity.** *PLoS One* 2011, **6**:e19016.

37. Thalanayar PM, Holguin F: **Rash, disseminated intravascular coagulation and legionella: Episode 10 and a rewind into the past**. *Respir Med Case Reports* 2015, **15**:95–100.

38. Roux D, Gaudry S, Dreyfuss D, El-Benna J, de Prost N, Denamur E, Saumon G, Ricard J-D: **Candida albicans impairs macrophage function and facilitates Pseudomonas aeruginosa pneumonia in rat.** *Crit Care Med* 2009, **37**:1062–7.

39. Chen AI, Dolben EF, Okegbe C, Harty CE, Golub Y, Thao S, Ha DG, Willger SD, O'Toole GA, Harwood CS, Dietrich LEP, Hogan DA: **Candida albicans ethanol stimulates Pseudomonas aeruginosa**

WspR-controlled biofilm formation as part of a cyclic relationship involving phenazines. *PLoS Pathog* 2014, **10**:e1004480.

40. Håkansson A, Kidd A, Wadell G, Sabharwal H, Svanborg C: **Adenovirus infection enhances in vitro adherence of Streptococcus pneumoniae.** *Infect Immun* 1994, **62**:2707–14.

41. Golda A, Malek N, Dudek B, Zeglen S, Wojarski J, Ochman M, Kucewicz E, Zembala M, Potempa J, Pyrc K: **Infection with human coronavirus NL63 enhances streptococcal adherence to epithelial cells.** *J Gen Virol* 2011, **92**(Pt 6):1358–68.

42. Teughels W, Sliepen I, Quirynen M, Haake SK, Van Eldere J, Fives-Taylor P, Van Ranst M: **Human cytomegalovirus enhances A. actinomycetemcomitans adherence to cells.** *J Dent Res* 2007, **86**:175–80.

43. Avadhanula V, Rodriguez CA, Devincenzo JP, Wang Y, Webby RJ, Ulett GC, Adderson EE: **Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner.** *J Virol* 2006, **80**:1629–36.

44. Rameix-Welti M-A, Zarantonelli ML, Giorgini D, Ruckly C, Marasescu M, van der Werf S, Alonso J-M, Naffakh N, Taha M-K: **Influenza A virus neuraminidase enhances meningococcal adhesion to epithelial cells through interaction with sialic acid-containing meningococcal capsules.** *Infect Immun* 2009, **77**:3588–95.

45. Okamoto S, Kawabata S, Terao Y, Fujitaka H, Okuno Y, Hamada S: **The Streptococcus pyogenes capsule is required for adhesion of bacteria to virus-infected alveolar epithelial cells and lethal bacterial-viral superinfection.** *Infect Immun* 2004, **72**:6068–75.

46. Jones WT, Menna JH, Wennerstrom DE: **Lethal synergism induced in mice by influenza type A virus and type Ia group B streptococci.** *Infect Immun* 1983, **41**:618–23.

47. Zavitz CCJ, Bauer CMT, Gaschler GJ, Fraser KM, Strieter RM, Hogaboam CM, Stampfli MR: **Dysregulated macrophage-inflammatory protein-2 expression drives illness in bacterial superinfection of influenza.** *J Immunol* 2010, **184**:2001–13.

48. Passariello C, Nencioni L, Sgarbanti R, Ranieri D, Torrisi MR, Ripa S, Garaci E, Palamara AT: **Viral hemagglutinin is involved in promoting the internalisation of Staphylococcus aureus into human pneumocytes during influenza A H1N1 virus infection.** *Int J Med Microbiol* 2011, **301**:97–104.

49. Aebi T, Weisser M, Bucher E, Hirsch HH, Marsch S, Siegemund M: **Co-infection of Influenza B and Streptococci causing severe pneumonia and septic shock in healthy women.** *BMC Infect Dis* 2010, **10**:308.

50. Kukavica-Ibrulj I, Hamelin M-E, Prince GA, Gagnon C, Bergeron Y, Bergeron MG, Boivin G: **Infection with human metapneumovirus predisposes mice to severe pneumococcal pneumonia.** *J Virol* 2009, **83**:1341–9.

51. Rubinas TC, Carey RB, Kampert MC, Alkan S, Lednicky JA: **Fatal hemorrhagic pneumonia concomitant with Chlamydia pneumoniae and parainfluenza virus 4 infection.** *Arch Pathol Lab Med* 2004, **128**:640–4.

52. Stewart PS, Franklin MJ: **Physiological heterogeneity in biofilms.** *Nat Rev Microbiol* 2008, **6**:199–210.

53. Yonezawa H, Osaki T, Kamiya S: **Biofilm Formation by Helicobacter pylori and Its Involvement for Antibiotic Resistance.** *Biomed Res Int* , **2015**:914791.

54. Van Ewijk BE, Wolfs TFW, Aerts PC, Van Kessel KPM, Fleer A, Kimpen JLL, Van der Ent CK: **RSV mediates Pseudomonas aeruginosa binding to cystic fibrosis and normal epithelial cells.** *Pediatr Res* 2007, **61**:398–403.

55. McNamara PS, Flanagan BF, Smyth RL, Hart CA: **Impact of human metapneumovirus and respiratory syncytial virus co-infection in severe bronchiolitis.** *Pediatr Pulmonol* 2007, **42**:740–3.

56. Avadhanula V, Wang Y, Portner A, Adderson E: **Nontypeable Haemophilus influenzae and Streptococcus pneumoniae bind respiratory syncytial virus glycoprotein.** *J Med Microbiol* 2007, **56**(Pt 9):1133–7.

57. Wilkinson TMA, Hurst JR, Perera WR, Wilks M, Donaldson GC, Wedzicha JA: **Effect of interactions between lower airway bacterial and rhinoviral infection in exacerbations of COPD.** *Chest* 2006, **129**:317–24.

58. Ishizuka S, Yamaya M, Suzuki T, Takahashi H, Ida S, Sasaki T, Inoue D, Sekizawa K, Nishimura H, Sasaki H: **Effects of rhinovirus infection on the adherence of Streptococcus pneumoniae to cultured human airway epithelial cells.** *J Infect Dis* 2003, **188**:1928–39.

59. Passariello C, Schippa S, Conti C, Russo P, Poggiali F, Garaci E, Palamara AT: **Rhinoviruses promote internalisation of Staphylococcus aureus into non-fully permissive cultured pneumocytes.** *Microbes Infect* 2006, **8**:758–66.

60. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, Iwasaki A: **Microbiota regulates immune defense against respiratory tract influenza A virus infection.** *Proc Natl Acad Sci U S A* 2011, **108**:5354–9.

61. Pettigrew MM, Gent JF, Revai K, Patel JA, Chonmaitree T: **Microbial interactions during upper respiratory tract infections.** *Emerg Infect Dis* 2008, **14**:1584–91.

62. Lysenko ES, Ratner AJ, Nelson AL, Weiser JN: **The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces.** *PLoS Pathog* 2005, **1**:e1.

63. Otero MC, Nader-Macías ME: **Inhibition of Staphylococcus aureus by H2O2-producing Lactobacillus gasseri isolated from the vaginal tract of cattle.** *Anim Reprod Sci* 2006, **96**:35–46.

64. Elahi S, Pang G, Ashman R, Clancy R: **Enhanced clearance of Candida albicans from the oral cavities of mice following oral administration of Lactobacillus acidophilus.** *Clin Exp Immunol* 2005, **141**:29–36.

65. OcaÃ±a VS, Pesce de Ruiz Holgado AãA, Nader-MacÃas ME: **Growth inhibition of Staphylococcus aureus by H 2 O 2 -producing Lactobacillus paracasei subsp. paracasei isolated from the human vagina**. *FEMS Immunol Med Microbiol* 1999, **23**:87–92.

66. Hogan DA, Kolter R: **Pseudomonas-Candida interactions: an ecological role for virulence factors.** *Science* 2002, **296**:2229–32.

67. Lopez-Medina E, Fan D, Coughlin LA, Ho EX, Lamont IL, Reimmann C, Hooper L V, Koh AY: **Candida albicans Inhibits Pseudomonas aeruginosa Virulence through Suppression of Pyochelin and Pyoverdine Biosynthesis.** *PLoS Pathog* 2015, **11**:e1005129.

68. Qin Z, Yang L, Qu D, Molin S, Tolker-Nielsen T: **Pseudomonas aeruginosa extracellular products inhibit staphylococcal growth, and disrupt established biofilms produced by Staphylococcus epidermidis.** *Microbiology* 2009, **155**(Pt 7):2148–56.

69. Kim Y, Mylonakis E: **Killing of Candida albicans filaments by Salmonella enterica serovar Typhimurium is mediated by sopB effectors, parts of a type III secretion system.** *Eukaryot Cell* 2011, **10**:782–90.

70. Héchard Y, Ferraz S, Bruneteau E, Steinert M, Berjeaud J-M: **Isolation and characterization of a Staphylococcus warneri strain producing an anti-Legionella peptide.** *FEMS Microbiol Lett* 2005, **252**:19–23.

71. KERR JR: **Inhibition of growth of fungi pathogenic to man by Stenotrophomonas maltophilia**. *J Med Microbiol* 1996, **45**:380–382.

72. Kreth J, Zhang Y, Herzberg MC: **Streptococcal antagonism in oral biofilms: Streptococcus sanguinis and Streptococcus gordonii interference with Streptococcus mutans.** *J Bacteriol* 2008, **190**:4632–40.

73. Tong H, Chen W, Shi W, Qi F, Dong X: **SO-LAAO, a novel L-amino acid oxidase that enables Streptococcus oligofermentans to outcompete Streptococcus mutans by generating H2O2 from peptone.** *J Bacteriol* 2008, **190**:4716–21.

74. Regev-Yochay G, Trzcinski K, Thompson CM, Malley R, Lipsitch M: **Interference between Streptococcus pneumoniae and Staphylococcus aureus: In vitro hydrogen peroxide-mediated killing by Streptococcus pneumoniae.** *J Bacteriol* 2006, **188**:4996–5001.

75. Wiley JA, Harmsen AG: **Pneumocystis infection enhances antibody-mediated resistance to a subsequent influenza infection.** *J Immunol* 2008, **180**:5613–24.

76. Dalton T, Dowd SE, Wolcott RD, Sun Y, Watters C, Griswold JA, Rumbaugh KP: **An in vivo polymicrobial biofilm wound infection model to study interspecies interactions.** *PLoS One* 2011, **6**:e27317.

77. Peters BM, Jabra-Rizk MA, O'May GA, Costerton JW, Shirtliff ME: **Polymicrobial interactions: impact on pathogenesis and human disease.** *Clin Microbiol Rev* 2012, **25**:193–213.

78. Duan K, Dammel C, Stein J, Rabin H, Surette MG: **Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication.** *Mol Microbiol* 2003, **50**:1477–91.

79. Harrison F: **Microbial ecology of the cystic fibrosis lung.** *Microbiology* 2007, **153**(Pt 4):917–23.

80. Gibson J, Sood A, Hogan DA: **Pseudomonas aeruginosa-Candida albicans interactions: localization and fungal toxicity of a phenazine derivative.** *Appl Environ Microbiol* 2009, **75**:504–13.

81. Davis-Hanna A, Piispanen AE, Stateva LI, Hogan DA: **Farnesol and dodecanol effects on the Candida albicans Ras1-cAMP signalling pathway and the regulation of morphogenesis.** *Mol Microbiol* 2008, **67**:47–62.

82. McAlester G, O'Gara F, Morrissey JP: **Signal-mediated interactions between Pseudomonas aeruginosa and Candida albicans.** *J Med Microbiol* 2008, **57**(Pt 5):563–9.

83. Hogardt M, Roeder M, Schreff AM, Eberl L, Heesemann J: **Expression of Pseudomonas aeruginosa exoS is controlled by quorum sensing and RpoS.** *Microbiology* 2004, **150**(Pt 4):843–51.

84. Méar J-B, Kipnis E, Faure E, Dessein R, Schurtz G, Faure K, Guery B: **Candida albicans and Pseudomonas aeruginosa interactions: More than an opportunistic criminal association?** *Médecine Mal Infect* 2013, **43**:146–151.

85. Hauser AR: **The type III secretion system of Pseudomonas aeruginosa: infection by injection.** *Nat Rev Microbiol* 2009, **7**:654–65.

86. Le Berre R, Nguyen S, Nowak E, Kipnis E, Pierre M, Quenee L, Ader F, Lancel S, Courcol R, Guery BP, Faure K: **Relative contribution of three main virulence factors in Pseudomonas aeruginosa pneumonia.** *Crit Care Med* 2011, **39**:2113–20.

87. Lau GW, Hassett DJ, Britigan BE: **Modulation of lung epithelial functions by Pseudomonas aeruginosa.** *Trends Microbiol* 2005, **13**:389–97.

88. Suga H, Smith KM: **Molecular mechanisms of bacterial quorum sensing as a new drug target**. *Curr Opin Chem Biol* 2003, **7**:586–591.

89. Miller MB, Bassler BL: **Quorum sensing in bacteria.** *Annu Rev Microbiol* 2001, **55**:165–99.

90. Diggle SP, Winzer K, Chhabra SR, Worrall KE, Cámara M, Williams P: **The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR.** *Mol Microbiol* 2003, **50**:29–43.

91. Rasmussen TB, Givskov M: **Quorum-sensing inhibitors as anti-pathogenic drugs**. *Int J Med Microbiol* 2006, **296**:149–161.

92. Méar J-B, Kipnis E, Faure E, Dessein R, Schurtz G, Faure K, Guery B: **Candida albicans and Pseudomonas aeruginosa interactions: more than an opportunistic criminal association?** *Médecine Mal Infect* 2013, **43**:146–51.

93. Saville SP, Lazzell AL, Monteagudo C, Lopez-Ribot JL: **Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of Candida albicans during infection.** *Eukaryot Cell* 2003, **2**:1053–60.

94. Sudbery PE: **Growth of Candida albicans hyphae.** *Nat Rev Microbiol* 2011, **9**:737–48.

95. Jacobsen ID, Wilson D, Wächtler B, Brunke S, Naglik JR, Hube B: **Candida albicans dimorphism as a therapeutic target.** *Expert Rev Anti Infect Ther* 2012, **10**:85–93.

96. Karkowska-Kuleta J, Rapala-Kozik M, Kozik A: **Fungi pathogenic to humans: molecular bases of virulence of Candida albicans, Cryptococcus neoformans and Aspergillus fumigatus**. 2009.

97. Fanning S, Mitchell AP: **Fungal biofilms.** *PLoS Pathog* 2012, **8**:e1002585.

98. Nicholls S, MacCallum DM, Kaffarnik FAR, Selway L, Peck SC, Brown AJP: **Activation of the heat shock transcription factor Hsf1 is essential for the full virulence of the fungal pathogen Candida albicans.** *Fungal Genet Biol* 2011, **48**:297–305.

99. Lingappa BT, Prasad M, Lingappa Y, Hunt DF, Biemann K: **Phenethyl alcohol and tryptophol: autoantibiotics produced by the fungus Candida albicans.** *Science* 1969, **163**:192–4.

100. Chen H, Fujita M, Feng Q, Clardy J, Fink GR: **Tyrosol is a quorum-sensing molecule in Candida albicans.** *Proc Natl Acad Sci U S A* 2004, **101**:5048–52.

101. Oh KB, Miyazawa H, Naito T, Matsuoka H: **Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in Candida albicans.** *Proc Natl Acad Sci U S A* 2001, **98**:4664–8.

102. Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, Dussault P, Nickerson KW: **Quorum sensing in the dimorphic fungus Candida albicans is mediated by farnesol.** *Appl Environ Microbiol* 2001, **67**:2982–92.

103. Hogan DA, Vik A, Kolter R: **A Pseudomonas aeruginosa quorum-sensing molecule influences Candida albicans morphology.** *Mol Microbiol* 2004, **54**:1212–23.

104. Dubern J-F, Diggle SP: **Quorum sensing by 2-alkyl-4-quinolones in Pseudomonas aeruginosa and other bacterial species.** *Mol Biosyst* 2008, **4**:882–8.

105. Costerton JW, Stewart PS, Greenberg EP: **Bacterial biofilms: a common cause of persistent infections.** *Science* 1999, **284**:1318–22.

106. Stewart PS: **Mechanisms of antibiotic resistance in bacterial biofilms.** *Int J Med Microbiol* 2002, **292**:107–13.

107. Stewart PS, Costerton JW: **Antibiotic resistance of bacteria in biofilms.** *Lancet* 2001, **358**:135–8.

108. Donlan RM, Costerton JW: **Biofilms: survival mechanisms of clinically relevant microorganisms.** *Clin Microbiol Rev* 2002, **15**:167–93.

109. Flemming H-C, Wingender J: **The biofilm matrix.** *Nat Rev Microbiol* 2010, **8**:623–33.

110. Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS, Stewart PS: **Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin.** *Antimicrob Agents Chemother* 2003, **47**:317–23.

111. Nichols WW, Dorrington SM, Slack MP, Walmsley HL: **Inhibition of tobramycin diffusion by binding to alginate.** *Antimicrob Agents Chemother* 1988, **32**:518–23.

112. Savage VJ, Chopra I, O'Neill AJ: **Staphylococcus aureus Biofilms Promote Horizontal Transfer of Antibiotic Resistance**. *Antimicrob Agents Chemother* 2013, **57**:1968–1970.

113. Lazăr V, Chifiriuc MC: **Medical significance and new therapeutical strategies for biofilm associated infections.** *Roum Arch Microbiol Immunol* , **69**:125–38.

114. Jefferson KK, Goldmann DA, Pier GB: **Use of confocal microscopy to analyze the rate of vancomycin penetration through Staphylococcus aureus biofilms.** *Antimicrob Agents Chemother* 2005, **49**:2467–73.

115. Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T: **Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes.** *Mol Microbiol* 2008, **68**:223–40.

116. Fux CA, Wilson S, Stoodley P: **Detachment characteristics and oxacillin resistance of Staphyloccocus aureus biofilm emboli in an in vitro catheter infection model.** *J Bacteriol* 2004, **186**:4486–91.

117. Hall-Stoodley L, Stoodley P: **Evolving concepts in biofilm infections.** *Cell Microbiol* 2009, **11**:1034–43.

118. Lewis K: **Persister cells: molecular mechanisms related to antibiotic tolerance.** *Handb Exp Pharmacol* 2012:121–33.

119. Hu Y, Coates A: **Nonmultiplying bacteria are profoundly tolerant to antibiotics.** *Handb Exp Pharmacol* 2012:99–119.

120. Chihara K, Matsumoto S, Kagawa Y, Tsuneda S: **Mathematical modeling of dormant cell formation in growing biofilm.** *Front Microbiol* 2015, **6**:534.

121. Lewis K: **Persister cells.** *Annu Rev Microbiol* 2010, **64**:357–72.

122. Schaible B, Taylor CT, Schaffer K: **Hypoxia increases antibiotic resistance in Pseudomonas aeruginosa through altering the composition of multidrug efflux pumps.** *Antimicrob Agents Chemother* 2012, **56**:2114–8.

123. Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS: **Oxygen limitation contributes to antibiotic tolerance of Pseudomonas aeruginosa in biofilms.** *Antimicrob Agents Chemother* 2004, **48**:2659–64.

124. Kindrachuk J, Scruten E, Attah-Poku S, Bell K, Potter A, Babiuk LA, Griebel PJ, Napper S: **Stability, toxicity, and biological activity of host defense peptide BMAP28 and its inversed and retro-inversed isomers.** *Biopolymers* 2011, **96**:14–24.

125. Taylor PK, Yeung ATY, Hancock REW: **Antibiotic resistance in Pseudomonas aeruginosa biofilms: towards the development of novel anti-biofilm therapies.** *J Biotechnol* 2014, **191**:121–30.

126. Lai S, Tremblay J, Déziel E: **Swarming motility: a multicellular behaviour conferring antimicrobial resistance.** *Environ Microbiol* 2009, **11**:126–36.

127. Rutherford ST, Bassler BL: **Bacterial quorum sensing: its role in virulence and possibilities for its control.** *Cold Spring Harb Perspect Med* 2012, **2**.

128. Coenye T, Nelis HJ: **In vitro and in vivo model systems to study microbial biofilm formation.** *J Microbiol Methods* 2010, **83**:89–105.

129. Lindsay D, von Holy A: **Bacterial biofilms within the clinical setting: what healthcare professionals should know.** *J Hosp Infect* 2006, **64**:313–25.

130. Stoodley P, Sauer K, Davies DG, Costerton JW: **Biofilms as complex differentiated communities.** *Annu Rev Microbiol* 2002, **56**:187–209.

131. Ramsugit S, Guma S, Pillay B, Jain P, Larsen MH, Danaviah S, Pillay M: **Pili contribute to biofilm formation in vitro in Mycobacterium tuberculosis.** *Antonie Van Leeuwenhoek* 2013, **104**:725–35.

132. Gil-Perotin S, Ramirez P, Marti V, Sahuquillo JM, Gonzalez E, Calleja I, Menendez R, Bonastre J: **Implications of endotracheal tube biofilm in ventilator-associated pneumonia response: a state of concept.** *Crit Care* 2012, **16**:R93.

133. Dimopoulos G, Poulakou G, Pneumatikos IA, Armaganidis A, Kollef MH, Matthaiou DK: **Short- vs long-duration antibiotic regimens for ventilator-associated pneumonia: a systematic review and meta-analysis.** *Chest* 2013, **144**:1759–67.

134. Capellier G, Mockly H, Charpentier C, Annane D, Blasco G, Desmettre T, Roch A, Faisy C, Cousson J, Limat S, Mercier M, Papazian L: **Early-onset ventilator-associated pneumonia in adults randomized clinical trial: comparison of 8 versus 15 days of antibiotic treatment.** *PLoS One* 2012, **7**:e41290.

135. Martin-Loeches I, Deja M, Koulenti D, Dimopoulos G, Marsh B, Torres A, Niederman MS, Rello J: **Potentially resistant microorganisms in intubated patients with hospital-acquired pneumonia: the interaction of ecology, shock and risk factors.** *Intensive Care Med* 2013, **39**:672–81.

136. Hermsen ED, Sullivan CJ, Rotschafer JC: **Polymyxins: pharmacology, pharmacokinetics, pharmacodynamics, and clinical applications.** *Infect Dis Clin North Am* 2003, **17**:545–62.

137. Flevari A, Theodorakopoulou M, Velegraki A, Armaganidis A, Dimopoulos G: **Treatment of invasive candidiasis in the elderly: a review.** *Clin Interv Aging* 2013, **8**:1199–208.

138. **Pseudomonas aeruginosa is not just in the intensive care unit any more: implications for empirical therapy. - PubMed - NCBI** [http://www.ncbi.nlm.nih.gov/pubmed/22425824]

139. Rathinakumar R, Walkenhorst WF, Wimley WC: **Broad-Spectrum Antimicrobial Peptides by Rational Combinatorial Design and High-Throughput Screening: The Importance of Interfacial Activity**. *J Am Chem Soc* 2009, **131**:7609–7617.

140. Pompilio A, Scocchi M, Pomponio S, Guida F, Di Primio A, Fiscarelli E, Gennaro R, Di Bonaventura G: **Antibacterial and anti-biofilm effects of cathelicidin peptides against pathogens isolated from cystic fibrosis patients.** *Peptides* 2011, **32**:1807–14.

141. Kaplan JB, LoVetri K, Cardona ST, Madhyastha S, Sadovskaya I, Jabbouri S, Izano EA: **Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci.** *J Antibiot (Tokyo)* 2012, **65**:73–7.

142. Yele AB, Thawal ND, Sahu PK, Chopade BA: **Novel lytic bacteriophage AB7-IBB1 of Acinetobacter baumannii: isolation, characterization and its effect on biofilm**. *Arch Virol* 2012, **157**:1441–1450.

143. Donlan RM: **Preventing biofilms of clinically relevant organisms using bacteriophage.** *Trends Microbiol* 2009, **17**:66–72.

144. Cerca N, Oliveira R, Azeredo J: **Susceptibility of Staphylococcus epidermidis planktonic cells and biofilms to the lytic action of staphylococcus bacteriophage K**. *Lett Appl Microbiol* 2007, **45**:313–317.

145. Hentzer M, Givskov M: **Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections.** *J Clin Invest* 2003, **112**:1300–7.

146. Raina S, Vizio DD, Odell M, Clements M, Vanhulle S, Keshavarz T: **Microbial quorum sensing: a tool or a target for antimicrobial therapy?** *Biotechnol Appl Biochem* 2009, **54**:65–84.

147. Wu H, Song Z, Hentzer M, Andersen JB, Molin S, Givskov M, Høiby N: **Synthetic furanones inhibit quorum-sensing and enhance bacterial clearance in Pseudomonas aeruginosa lung infection in mice.** *J Antimicrob Chemother* 2004, **53**:1054–61.

148. Rasmussen TB, Skindersoe ME, Bjarnsholt T, Phipps RK, Christensen KB, Jensen PO, Andersen JB, Koch B, Larsen TO, Hentzer M, Eberl L, Hoiby N, Givskov M: **Identity and effects of quorum-sensing inhibitors produced by Penicillium species.** *Microbiology* 2005, **151**(Pt 5):1325–40.

149. Skindersoe ME, Alhede M, Phipps R, Yang L, Jensen PO, Rasmussen TB, Bjarnsholt T, Tolker-Nielsen T, Høiby N, Givskov M: **Effects of antibiotics on quorum sensing in Pseudomonas aeruginosa.** *Antimicrob Agents Chemother* 2008, **52**:3648–63.

150. Kalia VC, Purohit HJ: **Quenching the quorum sensing system: potential antibacterial drug targets.** *Crit Rev Microbiol* 2011, **37**:121–40

Chapter 2

MATERIALS AND METHODS

2.1 Microorganisms and culture conditions

In this study, the bacterial species *P. aeruginosa* PAO1 and the fungal strain *C. albicans* SC5314 were used.

Both microorganisms were maintained in stock solutions with 20 % (V/V) glycerol at – 80 ± 2 °C. Every two weeks *C. albicans* and *P. aeruginosa* were subcultured from the frozen stock solutions onto Sabouraud Dextrose Broth (SDB; Liofilchem®, Roseto degli Abruzzi, Italy) and Tryptic Soy Broth (TSB; Liofilchem®) plates, respectively, supplemented with 1.2 % (W/V) agar (Liofilchem®) and incubated aerobically at 37 °C for 24-48 h.

2.1.1 Biofilm pre-inoculum

Before each experiment, a pre-inoculum was made by transferring some colonies from the subcultures to an flask containing TSB (for *P. aeruginosa*) or SDB (for *C. albicans*), being then incubated at 37 °C in an orbital shaker (120 rpm), overnight.

2.1.2 Biofilm formation assay

Biofilm assay was performed similar to the previously described methods [1]. To prepare biofilm inocula, each culture was diluted to achieve ~$1x10^7$ colony-forming unit (CFU)/mL. To adjust to the desired concentration, the initial cell suspension (pre-inoculum prepared in TSB/SDB) was centrifuge at 3000 *g* for 10 min and the pellet was resuspended in RPMI 1640 (Roswell Park Memorial Institute; Gibco® by Life Technologies) supplemented with sodium bicarbonate ($NaHCO_3$; Thermo Fisher Scientific, MA, USA) and buffered with morpholinepropanesulfonic acid (MOPS) (Affymetrix, Inc., Clevand, Ohio, USA). For *P. aeruginosa,* the optical density (OD) at 640 nm (OD_{640nm}) was read using an automated plate reader (Tecan Sunrise®) and the desired concentration was achieved by the following calibration curve CFU/mL = $6\times10^9 \times OD_{640nm} - 3\times10^8$, previously established by the research group. For *C. albicans,* the number of cells was counted with a Neubauer chamber (Marienfeld, Germany) and the initial cell concentration was calculated by the following formula CFU/mL = number of cells × 50 000 × dilution factor.

To promote biofilm formation, cell suspensions were grown on the surface of polystyrene, flat bottom 24 or 96-well microtiter plates (Orange Scientific, Brainel'Alleud, Belgium). Briefly, 1 mL (for 24-well plates) or 200 μL (for 96-well plates) of the standardized *P. aeruginosa* and *C. albicans*

suspension (1× 10^7 CFU/mL) in RPMI 1640 was allowed to adhere and form biofilms at 37 °C for 24 h. For dual-species cultures, the suspended inoculum of each species was combined in a 1:1 ratio. Negative controls consisted of wells filled with culture medium only (RPMI 1640 medium). Following biofilm formation, the cell supernatants were discarded, and the wells were washed twice with distilled sterile water.

2.2 Antimicrobial susceptibility

Different antimicrobial agents and QSI were tested throughout this work, in order to assess the effectiveness of novel and conventional antimicrobial strategies against single and polymicrobial consortia involving the aforementioned species.

For this study, the antibiotic used was PolyB (Biochrom, Berlin, Germany) and the antifungal agent tested was AmB (Sigma-Aldrich, St Louis, MO, USA), which were used combined.

Stock solutions of PolyB and AmB were prepared at 4500 mg/L and 10 mg/L in sterile water and in dimethyl sulfoxide (DMSO; Fisher Chemicals), respectively. Other antimicrobials were tested in single and mixed biofilms. All agents tested are shown in the following Table 5.

Table 5 All antimicrobials tested and respective concentration

		Antimicrobial	Concentration	Ref.
Conventional drugs		PolyB	8 mg/L	[2]
		AmB	0.016 mg/L	
QSI	**Commercial drugs**	Salicylic acid (Sigma)	200 mg/L	[3]
		Azithromycin (Sigma)	8 mg/L	[4]
		Ciprofloxacin (Sigma)	0.25 mg/L	[4, 5]
	Natural sources	Patulin (Sigma)	0.00025 mg/L	[6]
		Chlorogenic acid (Sigma)	750 mg/L	[7]

Table 5 (Continued) All antimicrobials tested and respective concentration

	Linalool (Sigma)	0.3 % (v/v)	[8]
	Farnesol (Sigma)	45 mg/L	[9]
Enzymes	Alginate lyase (Sigma)	20 U/L	[5]
	Desoxirribonuclease (GeneON, Minato, Tokyo, Japan)	100 mg/L	[10]

Stock solution of enzyme alginate lyase was prepared in phosphate-buffered saline (PBS) and desoxirribonuclease was prepared in PBS supplemented with magnesium chloride ($MgCl_2$; Fisher Chemicals). Stock solutions of all QSI were prepared in distilled sterile water, except for CIP and AZT were prepared in HCl (Fisher Chemicals), (1M) and DMSO respectively. All antimicrobial agents and QSI were stored at -20 °C before preparation stocks solutions. The concentrations of use were prepared in RPMI 1640 from stock solutions prior each experiment.

Biofilms (single- and dual-species) were formed as previously described in section 2.1.2. Afterwards, half the volume of suspension from each well culture medium was removed and an equal volume of the respective antimicrobial concentration was added to wells that were incubated for further 6 h (in case of biofilms treated for RNA extraction) or 24 h (in case of biofilms treated to be analyzed in terms of CFU and total biomass) at 37 °C in static conditions.

Each antimicrobial and QSI were tested for both *P. aeruginosa* and *C. albicans* in single and mixed biofilms and each antimicrobial was tested at least twice. Some of QSI and enzymes that did not any effect in biofilms was tested just once. The results were assessed using CFU and crystal violet (CV) staining method, as described next.

2.3 Methodologies

2.3.1 Biofilms analysis

2.3.1.1 Determination of cultivable cells

After washing biofilm cells, these were filled with NaCl 0.9 % (W/V) (J.T.Baker®) and a wells were scrapped to an eppendorf. To remove aggregates from the biofilm suspensions, epperdorfs were vigorously vortexed for 30 s. Then, the biofilm suspensions were serially 10-fold diluted in sterile distilled water, and 10 µL drops were plated onto TSA (for *P. aeruginosa*) and SDA (for *C.*

albicans) plates. For mixed cultures, selective growth media was used to count viable cells of each specific microorganism (SDA supplemented with 30 mg/L gentamicin (Sigma) for *C. albicans* and *Pseudomonas* Isolation Agar (PIA; Sigma) for specific isolation of *P. aeruginosa*). Agar plates were incubated aerobically at 37 °C for 24-48 h for cultivable cell counting. Values of cultivable sessile cells were expressed as $\log_{10}$ CFU per area (cm^2).

2.3.1.2 Biomass quantification

The mass of biofilms was determined using the crystal violet (CV) staining method firstly described by Christensen et al. [11] and adapted by Stepanović et al. [1]. CV is a basic dye, which binds to negatively charged molecules from the surface and to polysaccharides from the EPS [12]. Briefly, after biofilm formation, the planktonic fraction in the wells was discarded and the attached biofilms were washed twice and air dried. Subsequently, 200 μL of pure methanol (ChemLab, Zedelgem, Belgium) were transferred to each well for 15 min in order to fix the remaining attached bacteria. The plates were emptied and left to air dry again. Biofilms were then stained with 200 μL of 1 % (V/V) CV (Pro-Labs Diagnostics Inc.) for 5 min and were washed twice with sterile water. After this step the plates were air dried again and, at last, 200 μL of 33 % (V/V) of acetic acid (Thermo Scientific) were added to each well in order to solubilize the CV bound to the adherent bacteria. The quantitative analysis of biofilm production was performed through the measurement of optical density at 570 nm (OD_{570} nm) using an automated plate reader. Control experiments to avoid false results were also performed in order to determine whether the tested growth media and the plate material could absorb CV and interfere with biomass quantification.

2.3.1.3 Flow cytometry

Biofilm viability was also determined by flow cytometry as described below.

Initially the optimization of this process was made using planktonic cells of each microorganism *C. albicans* and *P. aeruginosa*. As it is known biofilms have a lot of variability so this step is only likely to be made using planktonic cells. In brief, biofilms preformed were washed twice, resuspended in 1 mL of phosphate-buffered saline (PBS) and vortexed at maximum speed (30 s). Lastly, were added 10 μL of SYTO BC (Thermo Scientific) at 50 μM and 2.5 μL of propidium iodide (PI) (Thermo Scientific) at 1.5 mM to 250 μL of suspension. After twenty minutes in the dark at room

temperature the cells were counted by EC800™ flow cytometer (SANYO, Moriguchi, Osaka, Japan). SYTO BC fluorescence was detected on the FL1 channel while PI fluorescence was detected on the FL4 channel. For all detected parameters, amplification was carried out using logarithmic scales. The concentration of bacteria was determined by acquiring the counts by the equipment. Multi-parametric analyses were performed on the scattering signals (forward scatter, FSC and side scatter, SSC), as well as on the FL1 (green fluorescence) and FL4 (red fluorescence).

2.3.2. Extraction of biofilm matrix

For extraction of the biofilm matrix, a previously in-group described protocol was followed. In brief, after washing and resuspending the biofilm,the biofilm suspension was sonicated for 30 s at 30 % amplitude in a sonicator (Cole-Parmer 750-Watt Ultrasonic Homogenizer with Temp Controller; IL, USA). The cells were separated from the matrix by centrifugation for 5 min at 5000 rpm. The supernatant was filtered with a filter of porosity 0.2 µm. The pellet corresponding to the cells of biofilm without matrix was resuspended in PBS to also be analyzed in the flow cytometer.

2.3.3. RNA extraction

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was isolated according the manufacturer's instructions, with minor modifications, when appropriate. The protocol has been optimized for the case of mixed biofilms in order to obtain the major yield possible for two microorganisms. This optimization was based on the efficacy of the mechanical and chemical lyses together (glass beads combined with phenol) and the easiness of the silica-membrane purification (RNA isolation column).

In brief, bacterial pellets were suspended in 500 µL of RLT lysis buffer plus 500 µL phenol solution (AppliChem, Darmstadt, Germany) and transferred into a 2 mL safe lock tube containing 0.5 g of glass beads (Sigma). Afterward, bacterial cell lyses was performed using a FastPrep®-24 cell disruptor (MP Biomedicals) at a 6.5 m/s during 35 s. The samples were then cooled on ice for 5 min and the lyses cycle repeated twice. After this, samples were centrifuged at 12000 *g* for 1 min, and supernatants transferred into a new tube and mixed with equal volume of 70% ethanol (Thermo Scientific). Thereafter, the samples were transferred into the RNA isolation column and centrifuged at 12000 *g* for 1 minute. The flow-through was discarded and each column washed with 700 µL of buffer RW1 and centrifuged at 8000 *g* for 1 minute. The flow-through was discarded and added

700 µL of buffer RPE and centrifuged at 8000 *g* for 1 minute. The flow-through was discarded and added again 500 µL of buffer RPE and centrifuged at 8000 *g* for 1 minute. The flow-through was discarded and the columns reinsert into a new collection tube for a new centrifugation at 12000 *g* for 2 minutes to remove any trace of the buffer RPE that contains ethanol. The collection tube was discarded and each column was inserted into a recovery tube. Finally, RNA was eluted by adding 30 µL of RNase-free water to the center of the membrane and centrifuged for 1 minute at 12000 *g*. The buffers used were provided by the kit extraction.

2.3.4. RNA yield and quality

The concentration and purity of the total RNA was spectrometrically assessed using a NanoDrop 1000™ (Thermo Scientific). The absorbance ratio A_{260}/A_{280} was used as an indicator of protein contamination, and A_{260}/A_{230} as an indicator of polysaccharide, phenol, and/or chaotropic salts contamination [13]. The best samples considering the ratios obtained were then analyzed by an external institution (i3S - Institute for Research and Innovation of the University of Porto) to access the integrity of the total RNA given by 23S/16S (for prokaryotes) and 28S/18S (for eukaryotes) rRNA ratio and the RNA quality indicator (RQI). RNA was stored at – 80 °C until further use.

2.3.5. RNA sequencing

RNA samples selected by its high quality (based on the aforementioned criteria) were sent to the BGI technologies Pvt. Ltd, a company specialized in transcriptomic analysis by RNA sequencing (RNA-seq).

2.4 Statistical analysis

Data were analyzed using the Prism software package (GraphPad Software version 6.01). Data were compared by one-way analysis of variance (ANOVA) and subsequent comparisons were performed using Turkey multiple-comparisons test. All tests were performed with a 95 % confidence level and differences were considered statistically significant at $*P<0.05$.

REFERENCES

1. Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M: **A modified microtiter-plate test for quantification of staphylococcal biofilm formation**. *J Microbiol Methods* 2000, **40**:175–179.

2. Rodrigues et al.: Fighting mixed bacterial-fungal biofilms in ventilator-associated pneumonia with antifungal-antibacterial combination therapy, submitted to PLos ONE.

3. Yang L, Rybtke MT, Jakobsen TH, Hentzer M, Bjarnsholt T, Givskov M, Tolker-Nielsen T: **Computer-aided identification of recognized drugs as Pseudomonas aeruginosa quorum-sensing inhibitors.** *Antimicrob Agents Chemother* 2009, **53**:2432–43.

4. Skindersoe ME, Alhede M, Phipps R, Yang L, Jensen PO, Rasmussen TB, Bjarnsholt T, Tolker-Nielsen T, Høiby N, Givskov M: **Effects of antibiotics on quorum sensing in Pseudomonas aeruginosa.** *Antimicrob Agents Chemother* 2008, **52**:3648–63.

5. Cotton LA, Graham RJ, Lee RJ: **The Role of Alginate in P. aeruginosa PAO1 Biofilm Structural Resistance to Gentamicin and Ciprofloxacin**. *J Exp Microbiol Immunol* , **13**:58–62.

6. Liaqat I, Bachmann RT, Sabri AN, Edyvean RGJ: **Isolate-Specific Effects of Patulin, Penicillic Acid and EDTA on Biofilm Formation and Growth of Dental Unit Water Line Biofilm Isolates**. *Curr Microbiol* 2010, **61**:148–156.

7. Annapoorani A, Umamageswaran V, Parameswari R, Pandian SK, Ravi AV: **Computational discovery of putative quorum sensing inhibitors against LasR and RhlR receptor proteins of Pseudomonas aeruginosa.** *J Comput Aided Mol Des* 2012, **26**:1067–77.

8. D'Auria FD, Tecca M, Strippoli V, Salvatore G, Battinelli L, Mazzanti G: **Antifungal activity of Lavandula angustifolia essential oil against Candida albicans yeast and mycelial form.** *Med Mycol* 2005, **43**:391–6.

9. Hassan Abdel-Rhman S, Mostafa El-Mahdy A, El-Mowafy M, Hassan Abdel-Rhman S, Mostafa El-Mahdy A, El-Mowafy M: **Effect of Tyrosol and Farnesol on Virulence and Antibiotic Resistance of Clinical Isolates of *Pseudomonas aeruginosa***. *Biomed Res Int* 2015, **2015**:1–7.

10. Tetz G V, Artemenko NK, Tetz V V: **Effect of DNase and antibiotics on biofilm characteristics.** *Antimicrob Agents Chemother* 2009, **53**:1204–9.

11. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH: **Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices.** *J Clin Microbiol* 1985, **22**:996–1006.

12. Li X, Yan Z, Xu J: **Quantitative variation of biofilms among strains in natural populations of Candida albicans.** *Microbiology* 2003, **149**(Pt 2):353–62.

13. Tavares L, Alves PM, Ferreira RB, Santos CN, Tan S, Yiap B, Raeymaekers L, Imbeaud S, Graudens E, Boulanger V, Barlet X, Zaborski P, Eveno E, Mueller O, Schroeder A, Auffray C, Bustin S, Nolan T, Wilson I, Rossen L, Norskov P, Holmstrom K, Rasmussen O, Esser K, Marx W, Lisowsky T, Sessions PF de, Dobrikova

E, Gromeier M, Rahmanto YS, et al.: **Comparison of different methods for DNA-free RNA isolation from SK-N-MC neuroblastoma**. *BMC Res Notes* 2011, **4**:3

Chapter 3

RESULTS AND DISCUSSION

3.1 Combined activity of AmB and PolyB against single- and mixed-species biofilms

In order to appraise the effect of combined AmB and PolyB in single- and mixed-species biofilms of *P. aeruginosa* or *C. albicans*, the consortia were formed for 24 h on 96-well plates and then treated for 24 h with the combination of both antimicrobial agents at 0.016 mg/L (AmB) and 8 mg/L (PolyB). Combined therapies can be useful to fight against polymicrobial cultures encompassing both bacterial and fungal species so these agents were tested in combination on mixed biofilms. The time of exposition and the concentration of those antimicrobial agents to treat *P. aeruginosa* and *C. albicans* consortia was previously determined (8 mg/L and 0.016 mg/L to PolyB and AmB respectively [1]. Afterwards, treated biofilms were characterized in terms of cultivable cells and total mass and compared with non-treated biofilms (Figure 5).

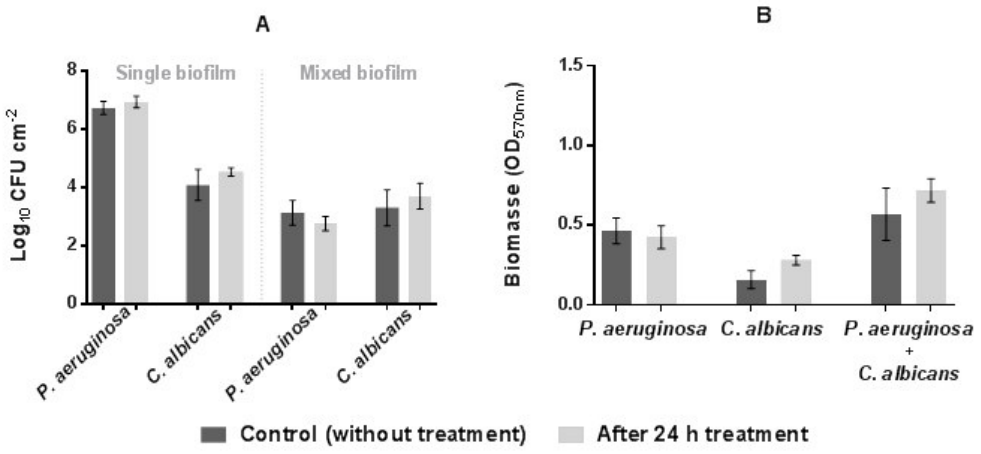


Figure 5 Characterization of single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans* in terms of (A) cultivable cells and (B) biomass, before and after treatment with the combination of AmB and PolyB at 0.016 mg/L and 8 mg/L respectively. Bars represent means ± standard deviations (sd) for at least two independent assays. * $P<0.05$ for statistic significant reductions.

Figure 5A showed that the combined action of AmB and PolyB could not reduce, in a significant extension, the number of cultivable cells of either *P. aeruginosa* or *C. albicans* in single- and in mixed-species biofilms. Contrariwise, Figure 5B demonstrated a slight increase for the biomass of both species, with exception for *P. aeruginosa*, in single biofilms. This increase in biomass could be eventually associated with the increase in the amount of EPS produced by the biofilm-associated cells when they were in contact with the antimicrobials.

Thus, results showed that the combined action of AmB and PolyB had no effect in inhibiting the formation of biofilms formed by *P. aeruginosa* and/or *C. albicans*. This inefficacy may be explained by the complex structure of biofilms formed by both pathogens. *C. albicans* strain SC5314 is highly

filamentous when compared with other strains of *C. albicans,* which is related with its virulence [2]. On the other hand, the use of RPMI 1640 culture medium to grow the biofilms, and particularly its compounds (such as L-glutamine) is known as one of the factors that induce germ tube production and, consequently, the hyphae growth in *C. albicans* yeast cells [3–5]. This can partly explain the ineffectiveness of the treatment with AmB and PolyB since hyphae filamentous create a complex structure difficulting the penetration of antimicrobials through the matrix and leads to antifungal resistance [6, 7].

A previous study determined that de minimum inhibitory concentration (MIC) of PolyB against *P. aeruginosa* strains isolated from mechanically ventilated patients isolates was 1 mg/L [8]. The Clinical & Laboratory Standards Institute (CLSI) provided guidelines for the susceptibility testing of polymyxins against *P. aeruginosa*: susceptibility, MIC≤2 mg/L; intermediate, MIC=4 mg/L; and resistance, MIC≥8 mg/L. In this study, it was used a concentration for PolyB of 8 mg/L. However, this concentration was still not effective to reduce the number of biofilm-encased cells. Therefore, higher dose is required to that applied in planktonic assays to observe an effect on biofilm cells. So, for the application of the susceptibility assays to bacterial cells encased in a biofilm, it becomes advantageous to determine the minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) [9]. However, in the literature, studies of antimicrobials are made recurrently in planktonic cells and this is reflected in a limitation for their investigation in biofilms.

In other study, AmB had a pronounced effect on biofilm inhibition (compared with other antifungal tested, the fluconazole). As such, the exposition of a 16 h-old biofilm to 4 mg/L of AmB resulted in >50 % inhibition in biofilm cells, whereas >80 % inhibition was detected for a higher dose of AmB (16 mg/L) [10]. The clinical drawback of this drug is related with its toxicity. In another study, it was observed that killing of biofilm cells was optimal at subtherapeutic concentrations of AmB (0.125 and 0.5 mg/L) [11].

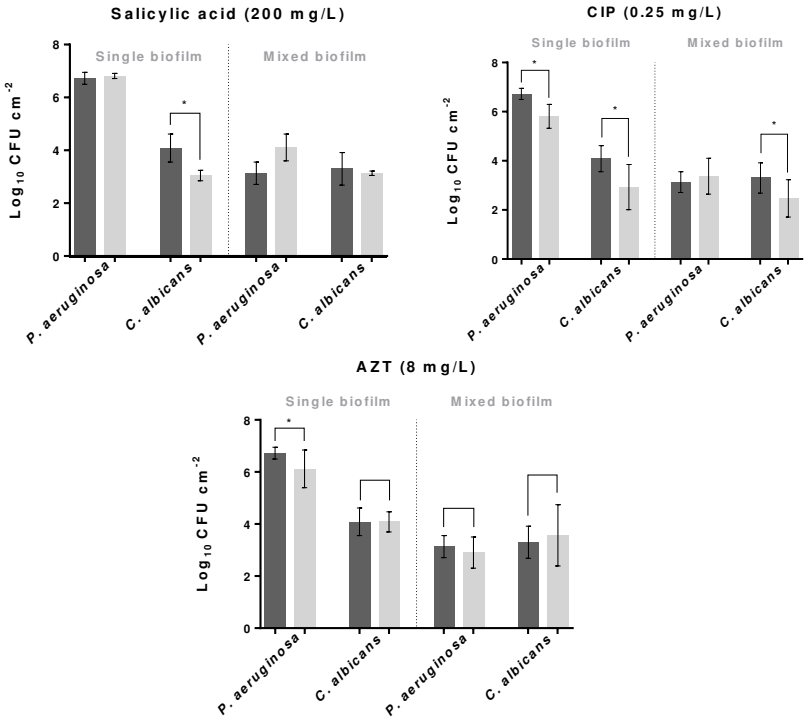
Maximizing the effectiveness and minimizing the toxicity of different antimicrobials is critical for clinical implications. Thus, these parameters are of extremely importance to better screen the therapeutic decisions in the treatment of VAP infections [11, 12].

3.2 Effect of QSI in single and mixed-species biofilms

Take into account the latest result, it was proposed to study new approaches and novel therapies for *P. aeruginosa* and *C. albicans* biofilm control. New strategies have been developed aiming to reduce or prevent biofilm formation and, when developed, interfering with intercellular communication among resident microorganism, thereby inhibiting QS mechanisms. A diverse range of compounds has been reported as QSI, therefore in this study, a screening of different agents was made in literature [13–17]. Among, different agents from distinct sources were selected, which included synthetic commercial inhibitors (AZT, CIP and salicylic acid) and compounds from natural sources (patulin, chlorogenic acid and farnesol). Moreover, two enzymes (alginate lyase and desoxirribonuclease) that have been reported as alternatives compared to the other QSI [18]. Salicylic acid, CIP and AZT are commercial synthetic drugs, with QS inhibition activity targeting N-acyl-l-homoserine lactone-mediated QS system in *P. aeruginosa*. Patulin, chlorogenic acid and farnesol, three important QS inhibitors obtained from natural sources, are known to interfere with AHL-mediated QS system in *P. aeruginosa* strains. Farnesol and linalool have also been selected as natural QSI, given their anti-QS in *C.albicans*. Among the compounds with enzymatic activity, alginate lyase and desoxirribonuclease were selected because in reducing biofilm growth [19].

After selecting the aforementioned agents, their efficiency was evaluated against single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans* (Figure 6).

COMMERCIAL DRUGS



NATURAL SOURCES

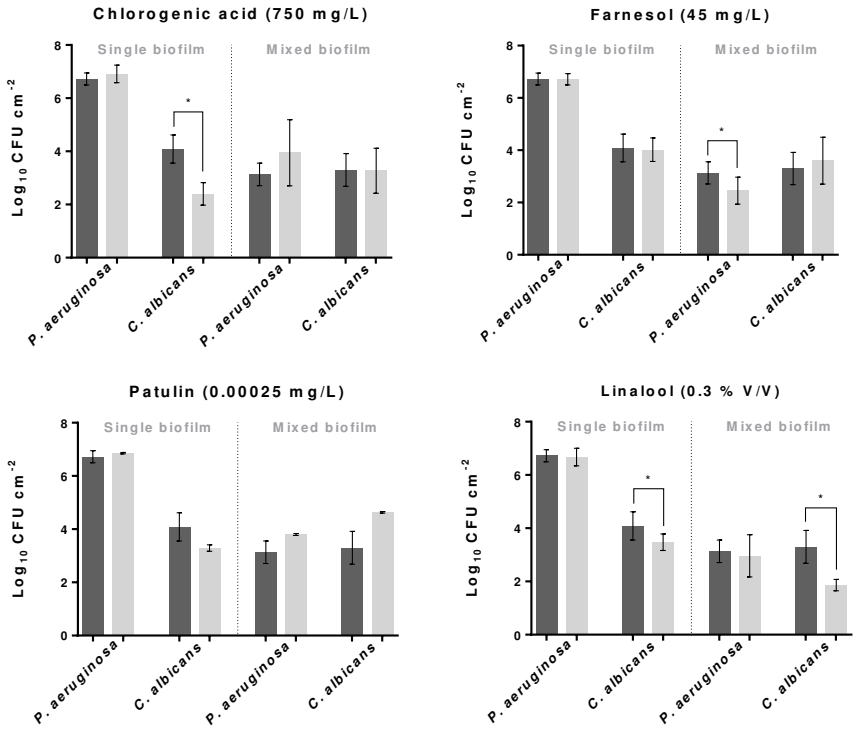


Figure 6 Effect of different alternative compounds in single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans*. Values of $\log_{10}$ CFU cm^{-2} were determined before and after 24 h treatment. Values represent means ± sd of at least two independent assays. Significant differences are represented with: *P<0.05.

ENZYMES

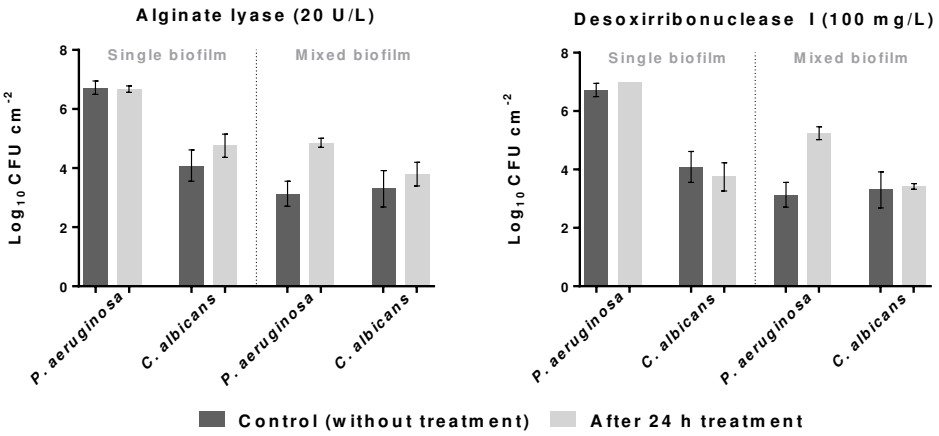
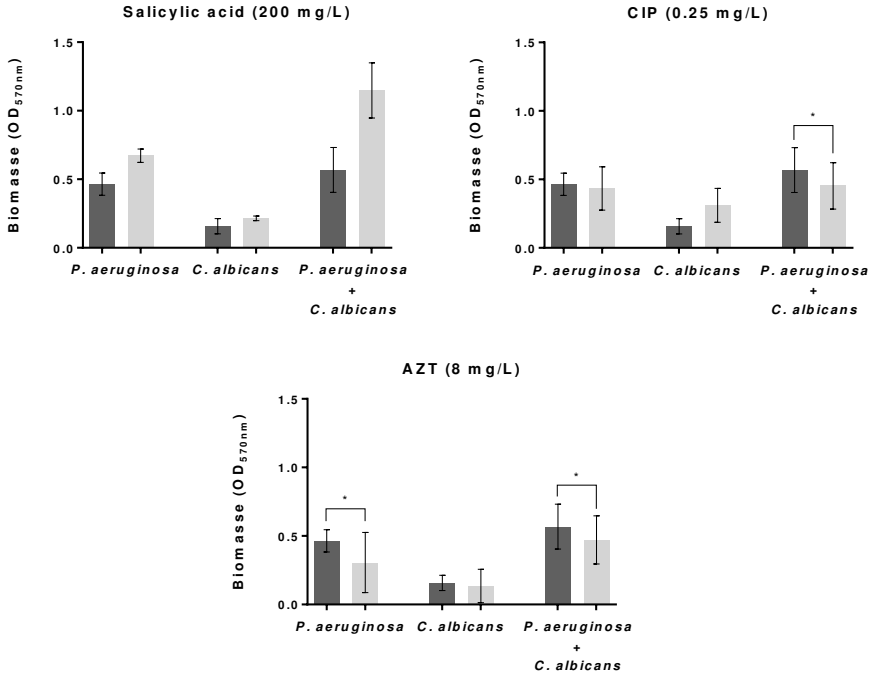


Figure 6 (Continued) Effect of different alternative compounds in single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans*. Values of $\log_{10}$ CFU cm^{-2} were determined before and after 24 h treatment. Values represent means ± sd of at least two independent assays. Significant differences are represented with: $*P<0.05$.

Results showed that *C. albicans* was the most affected species by the agents tested, in particular in single biofilms. It is still important to highlight that the significant reductions were observed in particular for single-species biofilms. These results suggest that when *P. aeruginosa* and *C. albicans* are in the same consortium, they can establish protective interations, making more treatment more difficult. These results were also observed by Roux et al. [20]. These investigators conclude that *C. albicans* airway colonization influences *P. aeruginosa* pneumonia prevalence.

Some QSI had no activity against biofilms and even slightly increase the values of CFU. It was the case of patulin, alginate lyase and desoxirribonuclease. Interestingly, CIP was the agent that could give the best results, by reducing the biofilm-encased cells of single- and mixed-species consortia. The effect of each QSI alone was further evaluated in the total mass of single and mixed-species biofilms (Figure 7).

COMMERCIAL DRUGS



NATURAL SOUCES

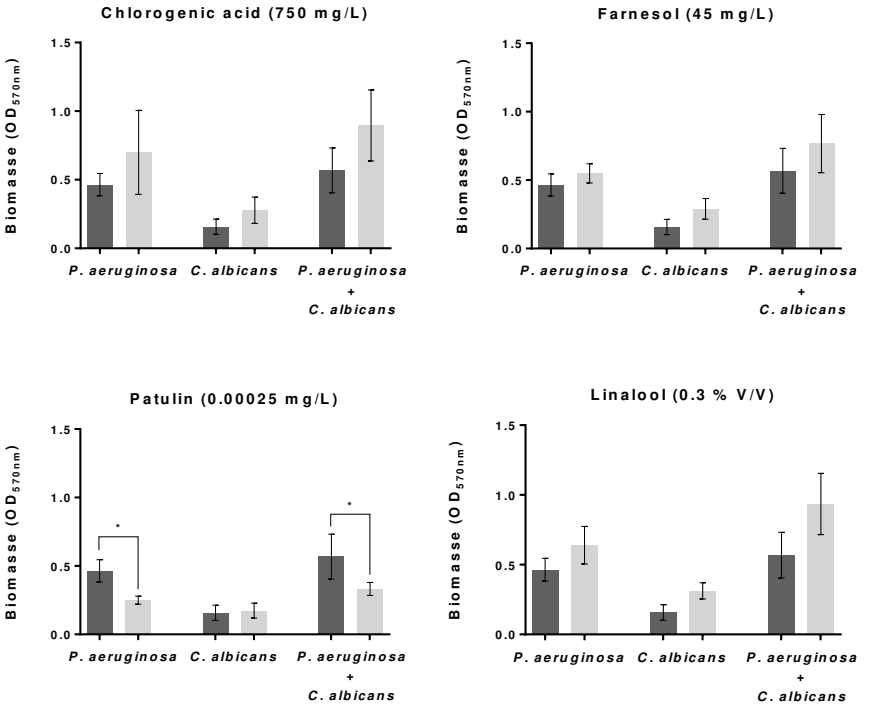


Figure 7 Effect of different alternative compounds in single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans*. Values of biomass were determined before and after 24 h treatment. Values represent means ± sd of at least two independent assays. Significant differences are represented with: * $P<0.05$.

ENZYMES

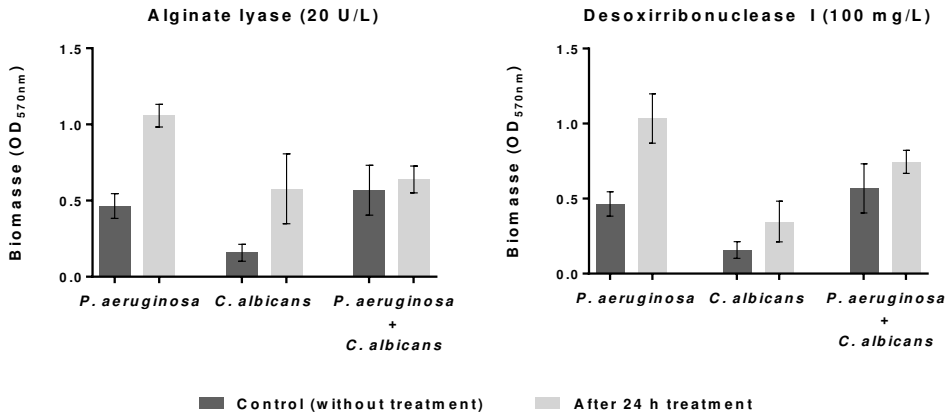


Figure 7 (Continued) Effect of different alternative compounds in single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans*. Values of biomass were determined before and after 24 h treatment. Values represent means ± sd of at least two independent assays. Significant differences are represented with: *P<0.05.

Regarding the effect in biofilm mass, most agents were ineffective in reducing the biomass of biofilms, with some of them even increasing it. Whereas *C. albicans* biomass was not disturbed by any agent, *P. aeruginosa* biomass was significantly decreased by AZT and patulin, which also promote significant changes in mixed biofilms biomass. Results of patulin that not shown any decrease in CFU but reduce significantly the biomass in *P. aeruginosa* and mixed biofilms can be explained by your possible interaction with the EPS. Only CIP, AZT and Patulin could lead statistic significant reduction in biomass. When *P. aeruginosa* biomass is affected, biomass of mixed biofilm also decrease, except for CIP that reduction was just observed for mixed biofilm.

In general, AZT is not used in the treatment of *P. aeruginosa* once it exhibits antipseudomonal activity (MIC ranging from 128 to 512 mg/L) [21]. More recent studies indicate that AZT and CIP may accomplish their beneficial action by preventing QS and reducing the pathogenicity of *P. aeruginosa* [13]. The same authors found MIC values of 800 mg/L for AZT and 12.5 mg/L for CIP. However, when the authors tested the minimal concentration of 8 mg/L of AZT were suppressed 71% of the 174 QS-regulated genes in *P. aeruginosa* PAO1 and 0.04 mg/L of CIP repressed 25 % of all QS genes. Another study has also suggested that inhibition of cell-cell communication is the mode of action by which AZT exerts its activity in *P. aeruginosa* infections [22], and still present activity against mature biofilm of the fungus [23]. In this study, AZT was effective against *P. aeruginosa* but failed in inhibiting *C. albicans* cells in single biofilms.

Interestingly, CIP showed also great activity against *C. albicans* and *P. aeruginosa* single biofilms, also disturbing mixed biofilms, in particular *C. albicans* cells. Whereas AZM exhibits a low level bactericidal or bacteriostatic effect against *P. aeruginosa*, CIP targets DNA gyrase (topoisomerase II) and thereby inhibits bacterial DNA synthesis leading to better results to treat infections.

In *C. albicans*, farnesol is known to accumulate in the extracellular medium to concentrations that repress the formation of hyphae [24, 25]. In other study was demonstrated that farnesol affected both the viability and antibiotic resistance in a number of bacterial and fungal species [26–28]. In case of *P. aeruginosa*, a recent study showed that its growth was inhibited by 30 % in the presence of 200 μM of farnesol [15]. In present study, for the same concentration, farnesol showed significant reduction in *P. aeruginosa* cells within mixed biofilms, about 22 % (compared with control) in total cultivable cells, however without significant disturbance in single consortia. However, this agent did not show any effect against *C. albicans.*

Another tested compound, salicylic acid, showed significant inhibition of QS-regulated gene expression and related phenotypes using 200 μg/ml of these inhibitor in *P. aeruginosa* [29]. These results suggest that the identified compounds have the potential to be used as antipathogenic drugs. However, no reduction was obtained with salicylic acid in *P. aeruginosa* biofilms, with the agent only leading to 25 % reduction in *C. albicans* single biofilms.

Linalool has been reported effective against biofilms of *C. albicans* [30–32]. According to D'Auria et al.[14], linalool inhibits *C. albicans* growth, with a MIC range from 0.09 – 0.29 % (V/V). In this study, a concentration of 0.3 % (V/V) linalool was used against *P. aeruginosa* and *C. albicans* biofilms, reducing significantly the number of *C. albicans*, 15 % and 44 % in single and mixed biofilms, respectively. For *P. aeruginosa,* a minimal reduction with greater emphasis, again, in mixed biofilms was observed.

3.3 Effect of double combination of QSI agents against single- and mixed-species biofilms

Based on the latest results, CIP can be consider the greater agent acting against *P. aeruginosa* and *C. albicans* biofilms. Following two different double combinations was tested (Figure 8 and 9). Considering the best result was a commercial drug (CIP), two different compounds of natural sources were added to minimize the use of antibiotics. In one case, it was added farnesol and other linalool to CIP. The advantage of using combination therapies is to have

a greater range of activity at the level of polymicrobial infections specially in fungal-bacterial biofilms.

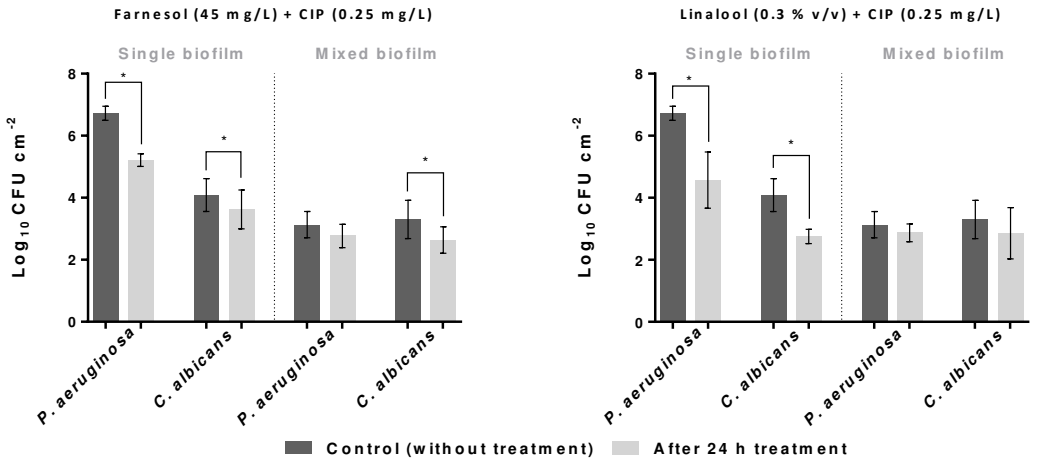


Figure 8 Effect of double combinations of QSI agents in single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans*. Values of $\log_{10}$ CFU cm^{-2} were determined before and after 24 h treatment. Values represent means ± sd of at least two independent assays. Significant differences are represented with: * $P<0.05$.

As observed, both antimicrobial combinations were effective against single-species biofilms of *P. aeruginosa* and *C. albicans*, significantly decreasing the number of cells of each organism in the biofilm, in particular for *C. albicans*. However, this disturbance was noticed particularly for linalool combined with CIP, leading to 2-log and 1-log reductions for *P. aeruginosa* and *C. albicans* biofilms, respectively. Regarding the effect on mixed-species biofilms, both treatments were effective in reducing biofilm-encased cells, with the combination farnesol+CIP promoting a significant reduction in *C. albicans* mixed-species biofilm.

Comparing the results from combined treatments with those obtained with the application of only one agent, it could be observed that combining CIP with either farnesol or linalool, led to similar results to that found for CIP alone. Therefore, in clinical and economic setting, it is not so advantageous to use two compounds instead of one. On the other hand, the combinatorial effect of farnesol with CIP also led to greater reductions that observed only for farnesol, and even the combination of CIP with linalool was more advantageous than using linalool alone. In fact, the effect on the reduction of viable cells increased in *P. aeruginosa* and *C. albicans* biofilms, compared with the results obtained for those compounds alone, in particular for single-species consortia.

Certain combinations of antimicrobials have exhibited synergistic effect against several pathogens [33]. It was defined as a significantly greater activity, the effect provided by two antimicrobials

agents combined in comparison with the effect provided by the sum of each antimicrobial agent alone [34]. It can be useful to obtain a best clinical therapeutic for treatment in patients with VAP. Regarding the effect of the combinations farnesol+CIP and linalool+CIP in biofilm mass (Figure 9), the results showed that the total mass of single and mixed-species biofilms of *P. aeruginosa* and *C. albicans* slightly decreased for most cases. An exception was observed for *C. albicans* in single biofilms when biofilms were treated with linalool+CIP combination. These results corroborate those obtained for cultivable cells (reduction in cultivable cells with decrease in total biomass). This decrease in biomass can be associated with the decrease in biofilm-associated cells when they were in contact with the antimicrobials.

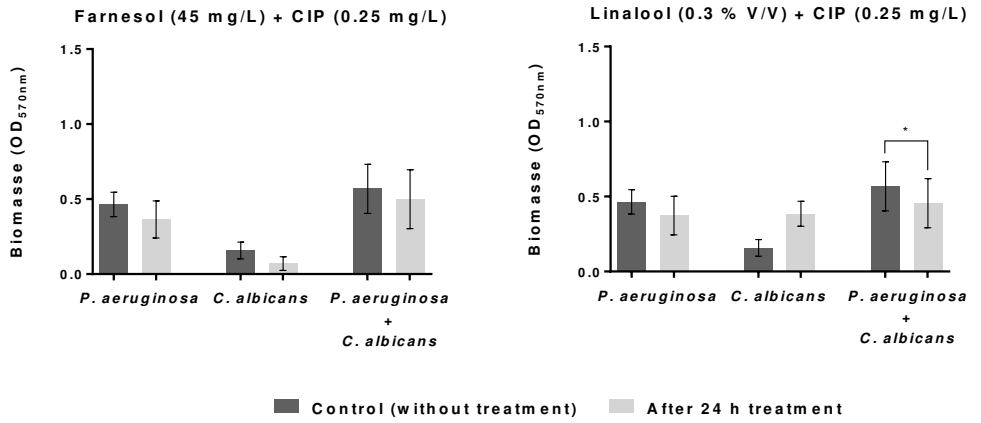


Figure 9 Effect of double combinations of QSI agents in single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans*. Values of biomass were determined before and after 24 h treatment. Values represent means ± sd of at least two independent assays. Significant differences are represented with: $*P<0.05$.

3.4 Effect of triple combination of QSI agents and conventional drugs against single- and mixed-species biofilms

Considering the results obtained to date, it was aimed to add a third compound to the antimicrobial combinations already tested. For this, chlorogenic acid, a compound of natural source that had shown any reduction in biofilm cells when tested alone was added to the combinations CIP+farnesol and CIP+linalool. To improve the effect of the combination of both conventional drugs AmB and PolyB, 4 different agents were added and the following combinations were tested on single- and mixed-species biofilms of PA and CA (AmB+PolyB+chlorogenic acid; AmB+PolyB+AZT; AmB+PolyB+CIP; AmB+PolyB+linalool).

The effect of triple combination of different agents was evaluated on single- and mixed-species biofilms (Figure 10).

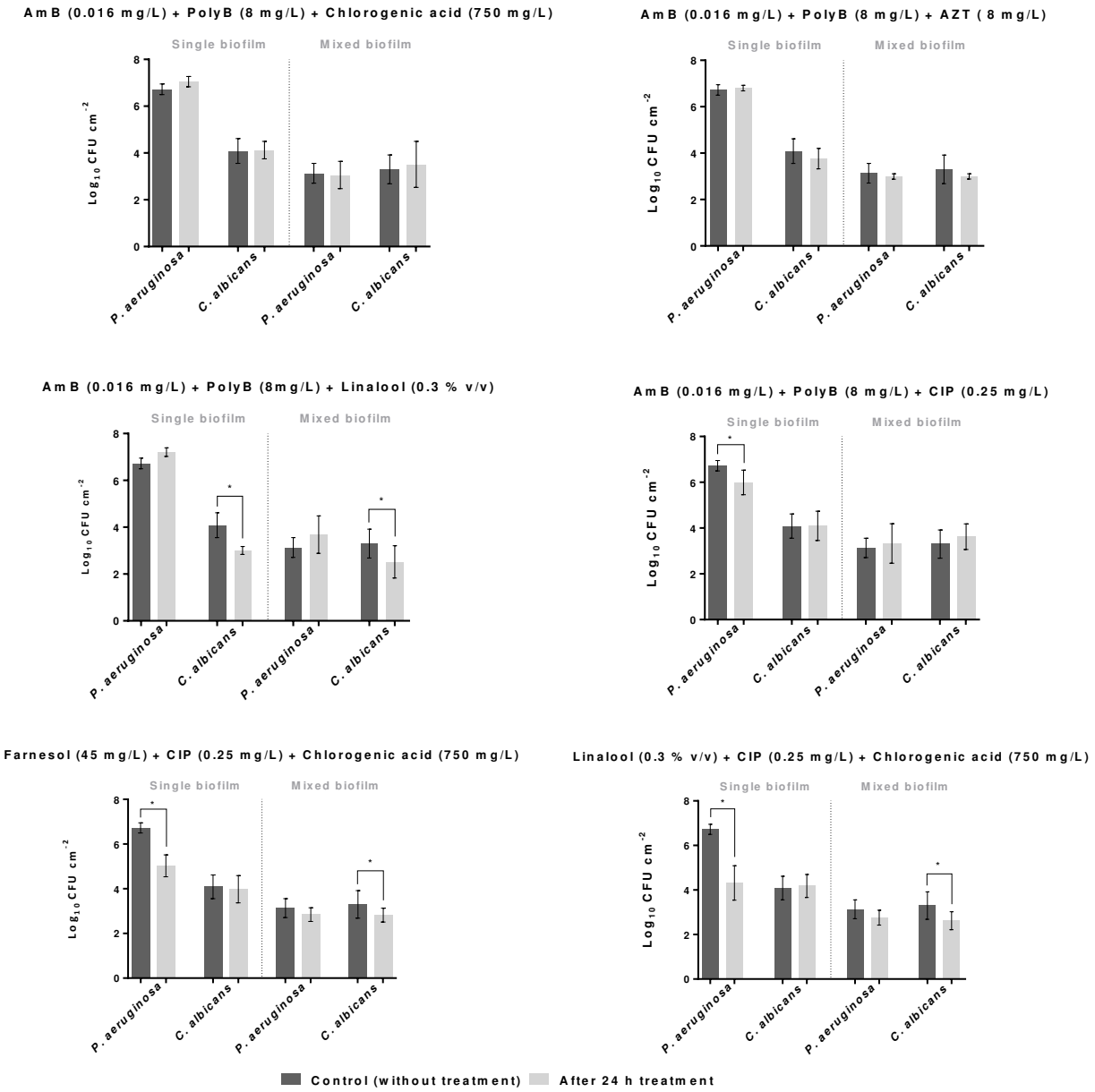


Figure 10 Effect of triples combinations of QSI agents and conventional drugs in single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans*. Values of $\log_{10}$ CFU cm^{-2} were determined before and after 24 h treatment. Values represent means ± sd of at least two independent assays. Significant differences are represented with: * $P<0.05$.

As can be seen by Figure 10, the reduction in the number of viable cells did not have large variations. Regarding the addition of chlorogenic acid to the farnesol+CIP and linalool+CIP combinations, the addition of third compound did not promote any significant reduction in biofilms, with the exception of *C. albicans* in dual-species consortia. In fact, the previous results had demonstrated that the combinatorial action of CIP+farnesol and CIP+linalool was effective in inhibiting *P. aeruginosa* and *C. albicans* in single-species biofilms. In this case, the addition of

chlorogenic acid had no effect or decrease (in some cases) in reducing *C. albicans* cells, which means that this agent has no effective against these pathogens. So it can be concluded that this inhibitor is not effective against *P. aeruginosa* and *C. albicans* and is more advantageous the use of dual combination.

Observing others combinations was concluded that the addition of CIP and linalool to AmB and PolyB led to significant reductions in *P. aeruginosa* and *C. albicans* single biofilms, respectively. Moreover, the number of cells of *C. albicans* in single and dual-species consortia was also reduced significantly when biofilms were treated with AmB+PolyB+linalool combination. Indeed, the aforementioned results had showed that AmB+PolyB dual combination could not disturb biofilms of *P. aeruginosa* and *C. albicans*. Therefore, CIP and linalool seemed to improve the effect of both antibiotics. Contrariwise, chlorogenic acid and AZT had no effect in disturbing single and mixed-species biofilms of *P. aeruginosa* and *C. albicans*, when combined with both conventional antibiotics.

Some combinations of antimicrobials have been suggested to have a synergistic behavior. It is the case of PolyB combined with AZT [35]. The same authors also investigated the combinations with time–kill method against 13 *P. aeruginosa* isolates and conclude that by addition of 4 mg/L AZT to PolyB (2 mg/L) produced a >2 log kill against most isolates and prevented regrowth in almost all isolates [35]. So, a minimal decrease observed in number of cells of mixed biofilms of *P. aeruginosa* treated with PolyB+AmB+AZT compared to obtain with PolyB+AmB can be explained by synergetic effect of PolyB and AZT.

The results obtained by triple combination of PolyB+AmB+CIP show better results than obtained before adding CIP for *P. aeruginosa* in single biofilm. This decrease in cells associated with biofilms may be associated with the of CIP because the results obtained for CIP when it was tested alone were better. Thus, using these combinatorial approach is not the better solution. In this situation using just CIP is more reasonable clinical and economically. However, previously studies, investigate that CIP in combination with PolyB had also a noticeable synergistic effect against *P. aeruginosa* [36] that was not observed in this work.

In other combination, AmB+PolyB+linalool, the same was observed, the result of linalool alone, in the general, had given better results compared with triple combination. Once again, the use of these combination in treatment of infection have economic and medical disadvantages.

The combinatorial effect of the aforementioned agents was further investigated in the biomass of *P. aeruginosa* and *C. albicans* biofilms (Figure 11).

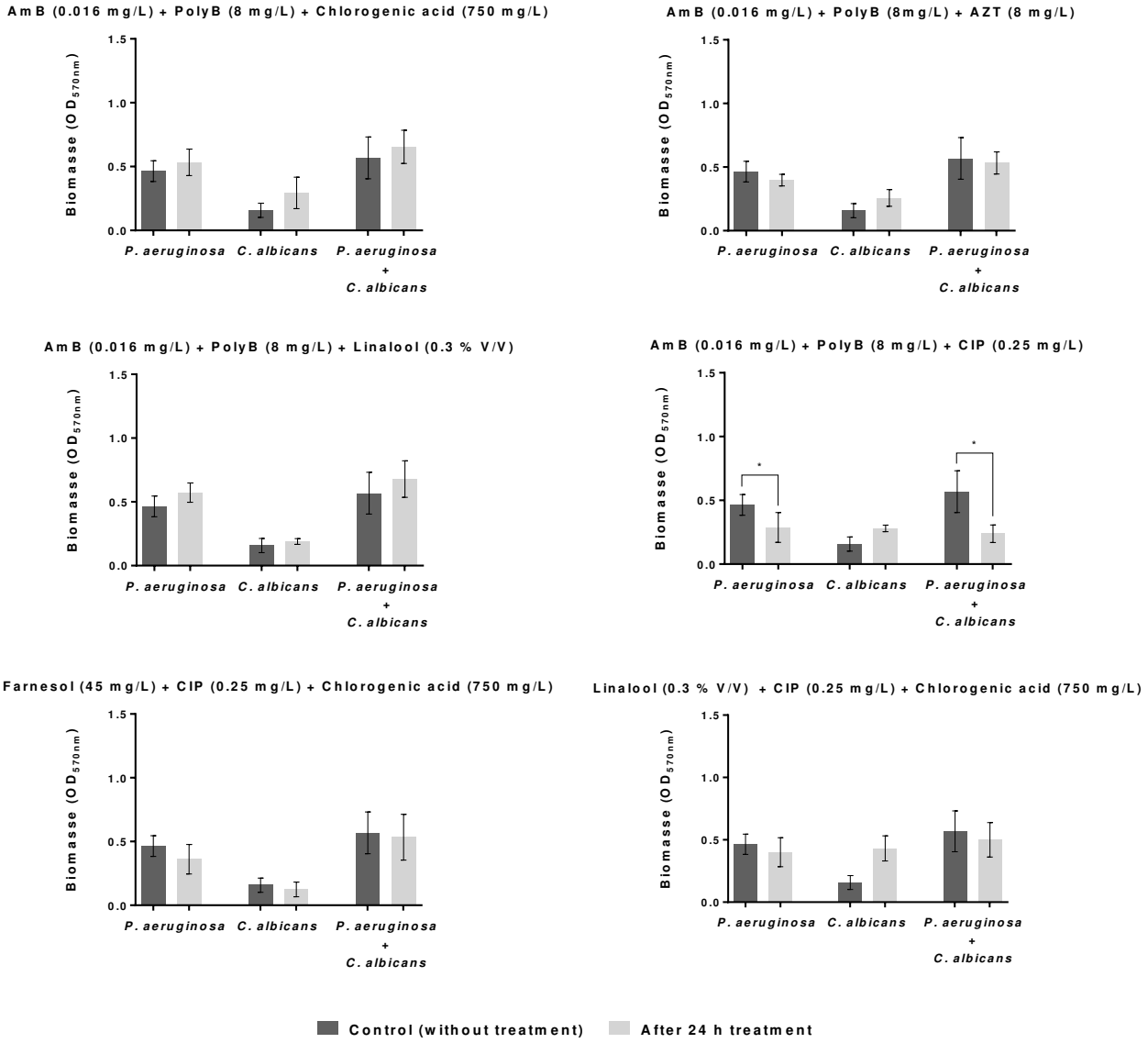


Figure 11 Effect of triples combinations of QSI agents and conventional drugs in single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans*. Values of biomass were determined before and after 24 h treatment. Values represent means ± sd of at least two independent assays. Significant differences are represented with: *P<0.05.

As observed, any treatment could significantly reduce the total biomass of *P. aeruginosa* and/or *C. albicans* biofilms. For example, for the combinations AmB+PolyB+chlorogenic acid and AmB+PolyB+linalool, the total mass of all the consortia was higher after treatment compared with the values before treatment (control). The only exception is to AmB+PolyB+CIP, which were reduced total biomass in single biofilm of *P. aeruginosa* and in mixed biofilm. In cultivable cells the reduce was just observed by single biofilm of *P. aeruginosa* so in case of mixed biofilm have a decrease in terms total biomass but have no changes in the number within the overall biofilms

indicate that these inhibitors could interfere eventually with the exopolysaccharide matrix of these biofilms but not with the cells. In general the trend persists: the results obtained in most inhibitors tested shown an increase biomass, but have no altered the number of cells within the biofilms. In other cases the number of viable cells decreased or remained unchanged and the biomass was higher. Based on these results it can be speculated that the pathogen may be in 'defense' response due to the presence of an external agent and produce more EPS. This outcome may have impact in the clinical context. The polymeric matrix of biofilms, formed for a mixture of polysaccharides, extracellular DNA, and proteins, acts as both a structural scaffold and a protective barrier to severe environments [37]. It can lead to an increase in the resistance of these biofilms against antimicrobials action.

In order to better understand the results obtained from the application of different agents against single and mixed-species biofilms of *P. aeruginosa* and *C. albicans*, an heat map was created based on the results obtained for cultivable cells (Figure 12).

This map allows a quick visualization the results of all combinations tested and easily identify those treatments which led to reductions in the number of cultivable cells according to the corresponding color. Red color corresponds to 0 log reduction or when there is no reduce in cells of biofilms after treatment, reflecting an ineffective treatment in the case of VAP infection. Green color corresponds to the maximum reduction obtained, in this case corresponds to 2.5 log, and although not very high values, considering the conditions which inhibitors were tested can be conclude that is a good reduction.

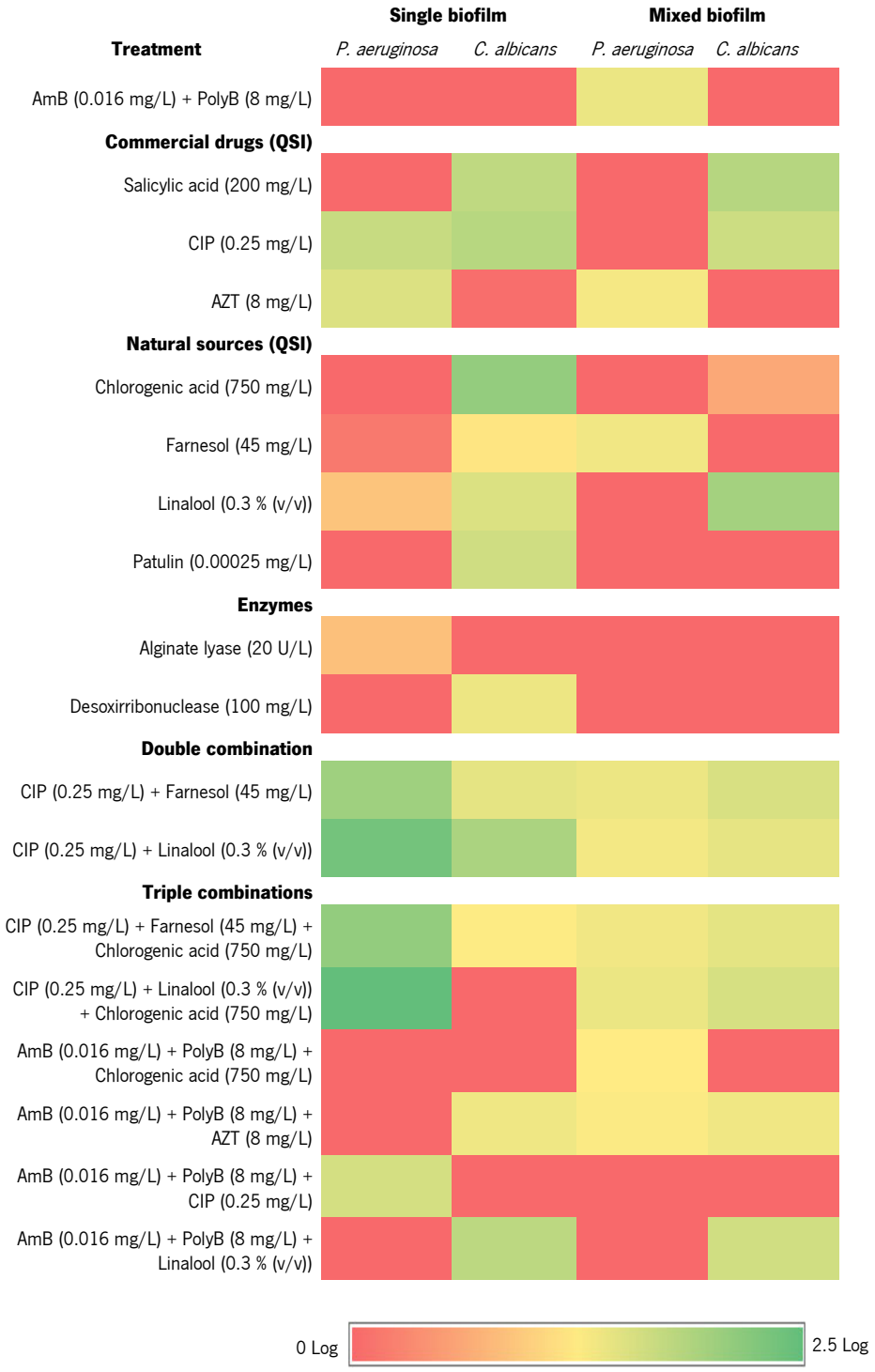


Figure 12 Heat map showing biofilm-associated cells reduction after 24 h treatment using single and combinatorial approaches.

3.5 Flow cytometry

In order to determine the number of total cells present in single and mixed biofilms and aiming to supplement the results obtained until this moment by others techniques, flow cytometry was used. This technique allows a quick achievement of these scores providing an overview about the type of cells that we have in our samples, namely its viability, size and complexity. In addition, this technique allows the discrimination between live and dead cells, providing also information about damaged cells [38]. Dot plots obtained for *P. aeruginosa* and *C. albicans* by flow cytometry are presented in Figure 13 and 14, respectively.

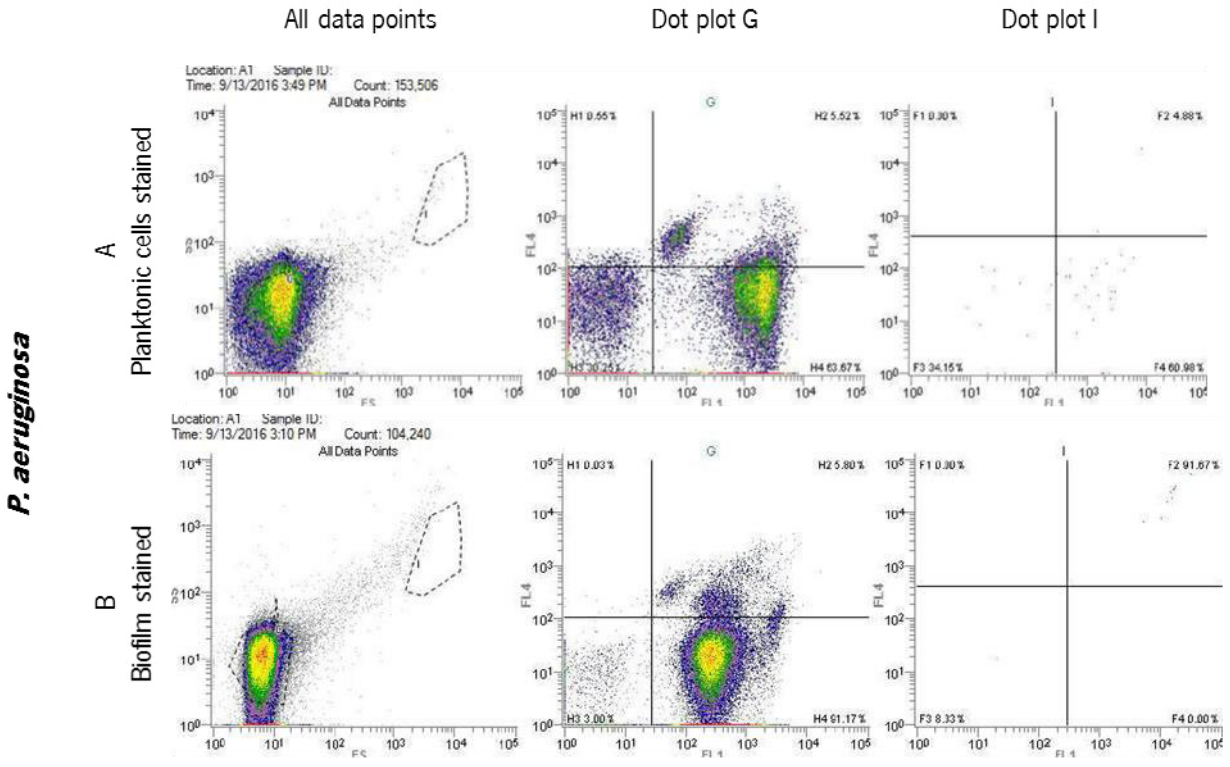


Figure 13 Schematic representation of dot plots obtained for *P. aeruginosa* by flow cytometry. Suspensions of cells were exposed to SYTO BC (detected on the FL1 channel) and (PI (detected on the FL4 channel). In 'all data points' are represented the dot plots SS (side scatter)×FS (forward scatter), (acquired in logarithm) delimited with areas G and I. These areas were defined to represent bacteria and fungi, respectively.

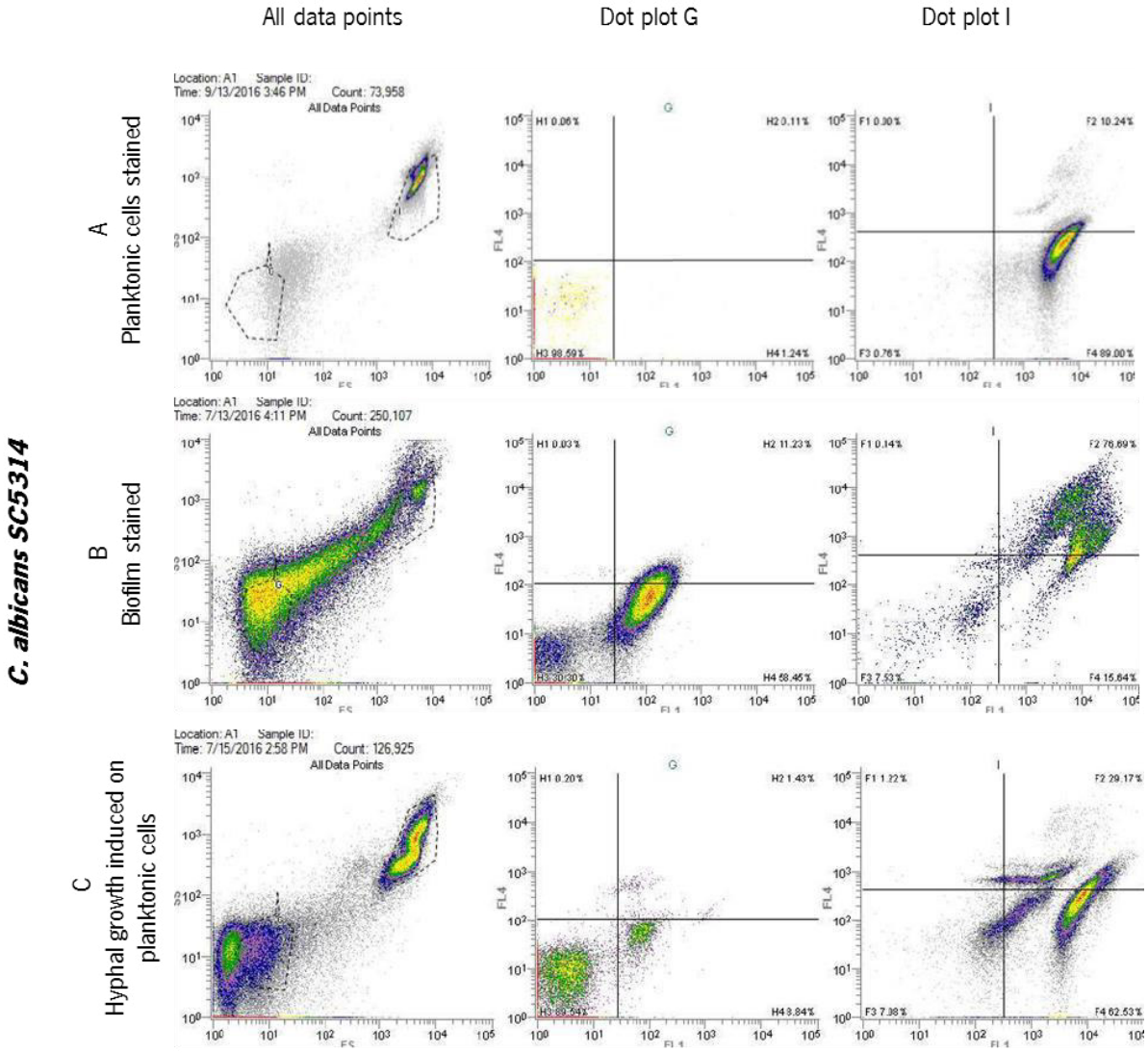


Figure 14 Schematic representation of dot plots obtained for *C. albicans* by flow cytometry. Suspensions of cells were exposed to SYTO BC (detected on the FL1 channel) and (PI (detected on the FL4 channel). In 'all data points' are represented the dot plots SS (side scatter)×FS (forward scatter), (acquired in logarithm) delimited with areas G and I. These areas were defined to represent bacteria and fungi, respectively.

Comparing the images of all data points by both pathogens *P. aeruginosa* and *C. albicans* of planktonic cells culture (represented as A in each figure) we can see that the region where they arise is very different, as to be expected since they have distinct sizes (*P. aeruginosa*: 0,6 x 2,0mm; *C. albicans*: 2-7 x 3-8 µm). As this technique allows a separation by size and complexity, it is possible separate these microorganisms when they are in a polymicrobial consortium using the same dyes.

After optimizing the experimental procedure, the biofilms analyses were assessed by quantifying the number of cells number on the samples.

At this stage, it was found that the behavior of the biofilm populations was different when compared with the planktonic populations (Figure 13B and 14B), particularly in the case of *C. albicans.*

Whereas with planktonic cells is possible define a typical region to each microorganisms, in biofilm cells it did not possible and there was a 'stain'.

According to these results two hypotheses were outlined: could a few hyphae of planktonic cells of *C. albicans* strain SC5314 compared to biofilm cells this affect the scores made by the instrument?; And/or the presence of biofilm matrix can cause this effect due to the different components displayed with different sizes and complexities?

In flow cytometry, cells are passed through the laser beam one at a time by a process known as hydrodynamic focusing. But if the sample not only has well-defined cells and has hyphal cells, it can be counted as more than one cell and lead to erroneous results. In order to discard this hypothesis, the possible influence of hyphae on the number of cell counts was assessed. For this, *C. albicans* strain SC5314 hyphal growth was induced on planktonic cells by growing them in RPMI + serum (2 % v/v) [5, 39] (Figure 14C). Simultaneously, we used another strain that does not has hyphae in both planktonic and biofilm cells (*C. albicans*, 547096) (Figure 15) to understand if hyphal growth interferes with flow cytometry acquisition. Analyzing the Figure 14C where is presented the hyphal growth induced in planktonic cells of *C. albicans* SC5314, the 'stain' is insignificant compared with obtained in biofilm. Also, examining the Figure 15B that correspond to biofilm of *C. albicans* 547096 without hyphae the 'stain' was again observed.

Regarding these results, two conclusions can be taken: the hyphal growth can have some influence on the counts (Figure 14C); despite this fact, these results did not justify entirely what happens when we have a biofilm, because when there are no hyphae it was also verified the presence of the 'stain' on the graph (Figure 15B).

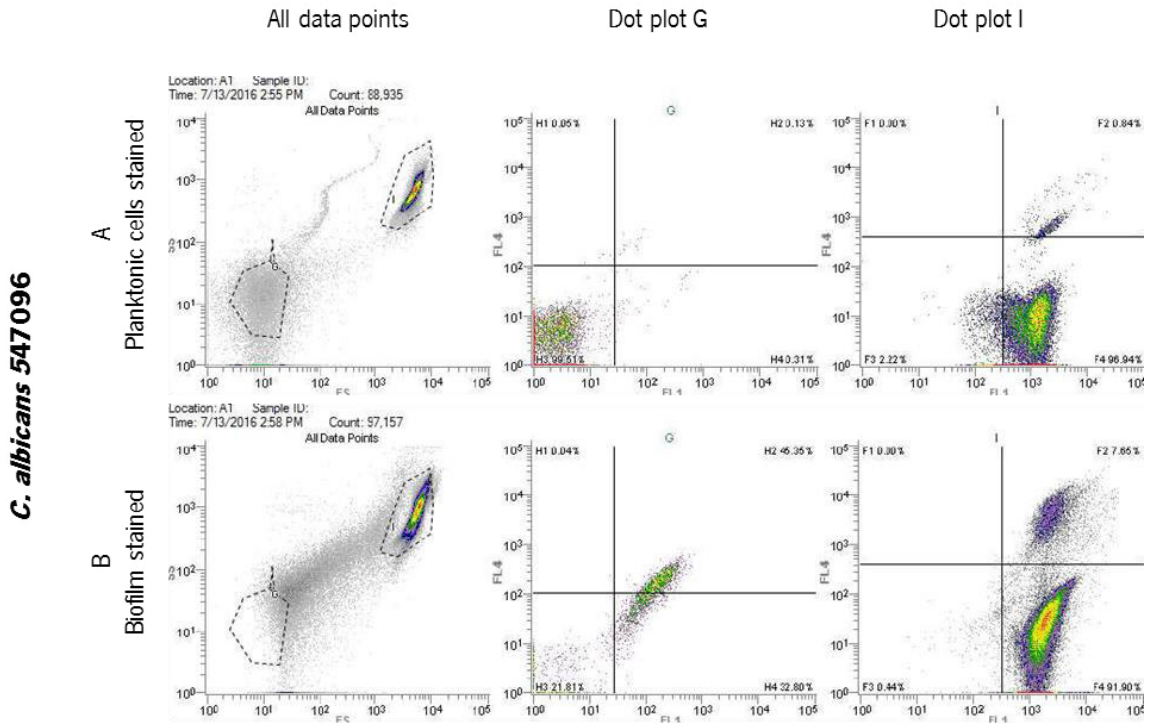


Figure 15 Schematic representation of dot plots obtained for *C. albicans* 547096 by flow cytometry. Suspensions of cells were exposed to SYTO BC (detected on the FL1 channel) and (PI (detected on the FL4 channel). In 'all data points' are represented the dot plots SS (side scatter)×FS (forward scatter), (acquired in logarithm) delimited with areas G and I. These areas were defined to represent bacteria and fungi, respectively.

Then, the next supposition was evaluated: the presence of biofilm matrix can influence the counts? The analysis was done with *C. albicans* strain 547096, so that the hyphae formation do not interfere with the results. Biofilm matrix was extracted using a sonication-based protocol (previously in-group described), in order to analyze the interference of the biofilm matrix with the flow cytometer. For that, were analyzed on the flow cytometer two different samples: biofilm cells without matrix and the biofilm matrix *per se*. The results obtained were presented in Figure 16 and 17.

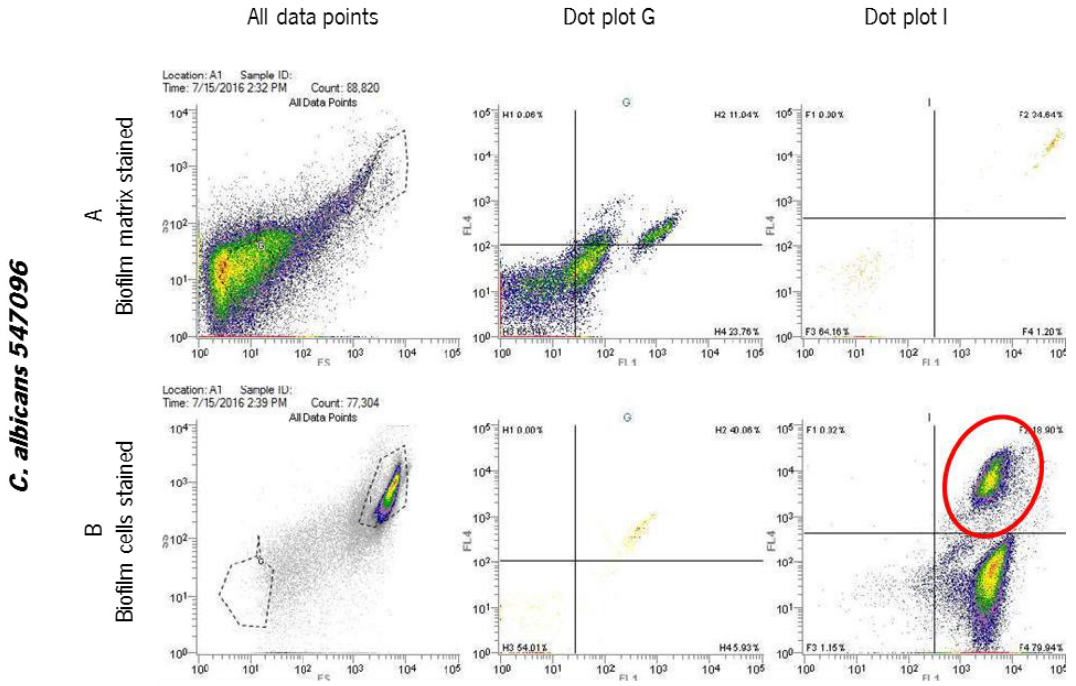


Figure 16 Schematic representation of dot plots obtained for *C. albicans* 547096 by flow cytometry. Suspensions of cells were exposed to SYTO BC (detected on the FL1 channel) and (PI (detected on the FL4 channel). In 'all data points' are represented the dot plots SS (side scatter)×FS (forward scatter), (acquired in logarithm) delimited with areas G and I. These areas were defined to represent bacteria and fungi, respectively.

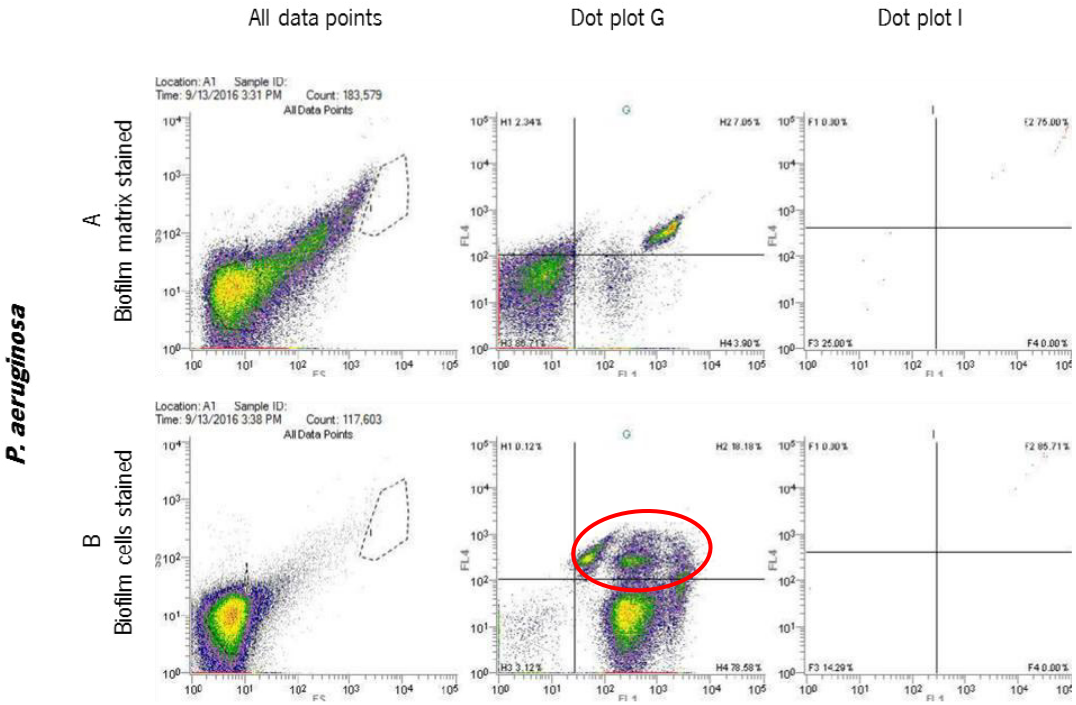


Figure 17 Schematic representation of dot plots obtained for *P.aeruginosa* by flow cytometry. Suspensions of cells were exposed to SYTO BC (detected on the FL1 channel) and (PI (detected on the FL4 channel). In 'all data points' are represented the dot plots SS (side scatter)×FS (forward scatter), (acquired in logarithm) delimited with areas G and I. These areas were defined to represent bacteria and fungi, respectively.

On Figures 16A and 17A it is demonstrated that when the biofilm matrix was analyzed in the flow cytometer showed a similar pattern than which was obtained previously in *C. albicans* biofilms. The complex polymeric matrix of biofilms is composed by a mixture of polysaccharides, eDNA and proteins [37], which have different sizes and complexities and when was passed in the equipment a wide range of different positions in the graph was obtained (Figures 16A and 17A).

Interestingly, by observing the graphs of biofilm cells without matrix it is possible to observe that the matrix extraction protocol used compromised cell viability (circled in red in Figure 16 and 17). We can see that this effect is more evident on *P. aeruginosa* due to the differences in cells of bacteria and fungus (usually bacteria require less time of sonication). Consequently, this process would still have to be optimized for the microorganisms in question. Probably, this problem should be overcome using others methods for extraction of biofilm matrix.

Flow cytometry is a very sensitive technique, therefore small variations in the sample will give a different dot plot (SS×FS). Consequently, it might be easy to understand the differences that we were getting over our work. As we can see in the images acquired, the graph points for yeast and bacteria occupy different positions on the dot-plot due to their different sizes. In fact, analyzing the same graphs (SS×FS) of distinct bacteria, it was shown that each has a characteristic profile [40], showing that the use of this technique has to be tailored to each studied microorganism.

Despite being a widely-used technique, there are few reports about biofilms analysis using flow cytometry. These reports usually present their data in graphs or tables, making it impossible to understand the effect of the biofilm matrix on these studies [41–44]. In fact, for bacteria the effect of biofilm matrix is not as notorious as in yeast, so this fact might have passed unnoticed by those who analyze this data.

3.6 RNA extraction

RNA extraction is an important and even crucial step for further transcriptomic analysis by RNA-seq. The goal was to analyze the two pathogens involved in this study: *P. aeruginosa* and *C. albicans* alone or in polymicrobial consortium. Briefly, RNA was isolated from independent biofilms (single and mixed consortia) and the results were evaluated if it was within the acceptable parameters to proceed to the analysis. RNA extraction is the first step and considered the most important, since the quality of RNA will influence the reproducibility and reliability of the subsequent applications [45]. Common indicators of RNA extraction success include the concentration, purity and integrity of RNA [46].

Table 6 presented the RNA yields and the ratios obtained for each extraction as well as the sample selected for consequent analysis according to these parameters.

Table 6 RNA yield and purity obtained for the RNA extractions performed. RNA extractions were made by using the RNeasy Mini kit (Qiagen)

Extraction number	Microorganism	Treatment (6h)	RNA yield (ng/ µl)	A_{260}/A_{280}	A_{260}/A_{230}	[28S:18S] or [23S:16S] ratio	RQI	RNA-seq analysis
4.1	*P. aeruginosa*	-	273.62	1.85	0.96	1.66	7.8	✓
4.2	*C. albicans*	-	69.74	1.97	1.33	1.51	8.9	✓
4.3	*P. aeruginosa + C. albicans*	-	113.50	1,84	0.79	1.37	7.7	✓
5.1	*P. aeruginosa*	-	180.96	1.85	1.32	1.78	7.8	✓
5.2	*C. albicans*	-	16.78	1.92	1.12	1.64	9.6	✓
5.3	*P. aeruginosa + C. albicans*	-	200.52	1.91	1.45	1.32	7.9	✓
6.1	*P. aeruginosa*	-	420.04	1.95	1.51	1.67	8.1	✓
6.2	*C. albicans*	-	64.13	1.43	0.26	1.66	8.6	✓
6.3	*P. aeruginosa + C. albicans*	-	173.71	1.76	0.85	1.50	8.6	✓
7.1	*P. aeruginosa*	-	77.00	1.65	0.68	1.26	7.4	✓
7.2	*C. albicans*	-	47.58	1.65	0.69	1.23	7.7	✓
7.3	*P. aeruginosa + C. albicans*	-	89.63	1.76	0.74	1.40	8.7	✓
8	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	129.50	1.95	1.27	1.26	8.0	✓
9	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	297.78	2.01	1.29	1.27	8.4	✓
10	*C. albicans*	-	231.80	2.25	0.81	1.21	9.6	✓
11	*P. aeruginosa*	-	58.18	2.16	0.70	2.91	N/A	×
12	*C. albicans*	-	1.14	2.33	0.18	1.11	N/A	×
13	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	94.30	2.04	0.60	1.64	8.1	✓
14	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	18.79	1.94	0.27	1.73	3.5	×
15	*P. aeruginosa + C. albicans*	-	31.72	1.40	0.35	0.77	4.4	×
16	*P. aeruginosa + C. albicans*	-	59.11	1.61	0.65	1.28	8.4	✓
17	*P. aeruginosa*	-	60.22	1.81	1.30	1.56	8.2	✓

18	*C. albicans*	-	57.90	1.78	1.43	0.83	3.4	✗
19	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	952.17	2.00	1.11	1.85	8.0	✓
20	*P. aeruginosa + C. albicans*	-	110.80	1.75	0.62	1.51	3.2	✗
21	*P. aeruginosa*	-	171.13	1.68	0.83	1.57	8.2	✓
22	*C. albicans*	-	50.27	1.48	0.58	0.42	3.1	✗
23	*P. aeruginosa + C. albicans*	-	433.23	2.08	0.91	1.77	7.9	✓
24	*C. albicans*	-	87.83	2.07	1.26	1.05	6.8	✓
25	*P. aeruginosa*	-	481.83	2.05	1.97	1.72	8.4	✓
26	*C. albicans*	-	201.66	2.01	1.94	0.85	2.0	✗
27	*P. aeruginosa + C. albicans*	-	218.64	1.92	1.46	1.75	7.7	✓
28	*C. albicans*	-	305.87	2.17	1.71	1.14	7.3	✓
29	*P. aeruginosa + C. albicans*	-	585.59	2.29	1.26	1.51	7.6	✓
30	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	975.11	2.19	1.76	1.61	8.2	✓
31	*P. aeruginosa*	-	967.13	1.95	5.64	1.65	8.9	✓
32	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	524.08	1.84	1.47	1.50	8.4	✓
33	*P. aeruginosa*	-	868.78	1.98	1.92	1.86	9.0	✓
34	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	642.39	1.98	1.70	1.67	8.1	✓
35	*P. aeruginosa + C. albicans*	-	353.44	1.95	2.20	1.81	7.8	✓
36	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	573.28	1.93	1.54	1.49	8.1	✓
37	*P. aeruginosa*	-	744.08	2.14	1.93	1.61	9.2	✓
38	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	163.15	2.13	1.49	1.56	8.1	✓
39	*P. aeruginosa + C. albicans*	-	252.10	2.28	1.95	1.67	7.8	✓
40	*C. albicans*	-	74.13	2.21	1.12	0.85	2.9	✗
41	*C. albicans*	-	22.78	1.94	1.44	0.93	10	✗
42	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L) + Chlorogenic acid (750 mg/L)	336.51	1.88	1.75	1.53	8.0	✓
43	*C. albicans*	-	112.79	2.29	1.88	0.67	3.7	✗

44	*C. albicans*	-	103.06	2.23	2.14	1.02	3.2	✗
45	*C. albicans*	-	22.68	1.97	1.77	0.73	1.7	✗
46	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L) + Chlorogenic acid (750 mg/L)	423.54	2.21	N/A	1.64	8.4	✓
47	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L) + Chlorogenic acid (750 mg/L)	666.81	2.08	1.79	1.75	8.5	✓
48	*C. albicans*	-	63.41	2.20	1.73	0.80	6.0	✗
49	*C. albicans*	-	188.94	1.92	1.43	0.93	6.4	✓
50	*C. albicans*	-	187.82	2.21	1.81	0.96	3.2	✗
51	*C. albicans*	-	125.99	2.04	0.22	1.12	6.9	✓
52	*C. albicans*	-	291.78	2.39	1.63	1.03	7.1	✓
53	*P. aeruginosa + C. albicans*	-	441.25	2.25	1.37	1.77	7.8	✓
54	*C. albicans*	-	120.34	2.35	2.59	1.05	2.8	✗
55	*P. aeruginosa + C. albicans*	-	214.96	2.32	2.30	1.82	7.8	✓
56	*C. albicans*	-	139.66	2.23	1.96	0.99	3.7	✗
57	*C. albicans*	-	81.30	1.81	2.24	0.35	2.7	✗
58	*C. albicans*	-	57.30	1.36	0.32	-	-	✓
59	*P. aeruginosa + C. albicans*	-	266.40	1.65	N/A	-	-	✓
60	*P. aeruginosa + C. albicans*	-	98.40	2.15	1.19	-	-	✓
61	*P. aeruginosa + C. albicans*	-	108.40	2.07	1.54	-	-	✓
62	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	382.60	1.87	1.36	-	-	✓
63	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	339.50	1.66	N/A	-	-	✓
64	*P. aeruginosa + C. albicans*	-	309.40	2.21	0.90	-	-	✓
65	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	435.10	1.93	1.74	-	-	✓
66	*P. aeruginosa + C. albicans*	PolyB (8 mg/L) + AmB (0.016 mg/L)	378.50	1.99	1.28	-	-	✓

Not all extractions performed have enough quality to continue for analysis, as can be seen in Table 6. Therefore some samples were excluded *a priori* to not affect the reliability of the downstream results. By analysis of the samples excluded is important underline that for *C. albicans* is more difficult obtain good yield and purity compared with *P. aeruginosa*. In case of samples from polymicrobial biofilms although a good yield has been achieved, the purity parameters were not considered acceptable, probably due the contamination with *C. albicans*.

3.7 RNA sequencing

RNA-seq is an important tool to transcriptome profiling that uses deep-sequencing technologies. In this point the main goal was to evaluate the differences in the genome of two pathogens involved in this study (*P. aeruginosa* and *C. albicans*), again, when they are alone or together. This is important to understand the differences of their behavior when they are alone or in a polymicrobial consortium and understand the result of their interaction. To make this comparative analyze, RNA was extracted from different conditions: single biofilms of *P. aeruginosa* and *C. albicans* and mixed biofilms. RNA was also extracted from the mixed biofilm treated with AmB and PolyB and still with AmB, PolyB and chlorogenic acid.

After carry out the extractions and evaluate the samples with quality, firstly the construction of libraries was made in home but the possibly some component of the kit was damaged and could not get results. At a later stage, RNA samples were sent to an outside company (BGI technologies Pvt. Ltd) to do sequencing. Unfortunately, the results of extraction did not meet all requirements to be considered good samples and then proceed with the analysis. In Annex I it can be seen the results sent by the company.

There were some conditions that met all the requirements for a good analysis (e.g.: *P. aeruginosa*) but the absence of a point of comparison led us to not continue the analysis.

Although the samples were sent in the storage conditions required (in dry ice in an isothermal box) this was not enough for them to get there intact. This is probably because the samples took several days to arrive to the company (approximately 10 days). In a further analysis it will need to be taken into account.

REFERENCES

1. Rodrigues, et al.: Fighting mixed bacterial-fungal biofilms in ventilator-associated pneumonia with antifungal-antibacterial combination therapy, submitted to PLOS ONE.

2. Hua X, Yuan X, Mitchell BM, Lorenz MC, O'Day DM, Wilhelmus KR: **Morphogenic and genetic differences between Candida albicans strains are associated with keratomycosis virulence.** *Mol Vis* 2009, **15**:1476–84.

3. Greene JN: **: *Candida* and Candidiasis**. *Clin Infect Dis* 2002, **35**:498–499.

4. Berman J: **Morphogenesis and cell cycle progression in Candida albicans.** *Curr Opin Microbiol* 2006, **9**:595–601.

5. Kucharíková S, Tournu H, Lagrou K, Van Dijck P, Bujdáková H: **Detailed comparison of Candida albicans and Candida glabrata biofilms under different conditions and their susceptibility to caspofungin and anidulafungin.** *J Med Microbiol* 2011, **60**(Pt 9):1261–9.

6. Douglas LJ: **Candida biofilms and their role in infection.** *Trends Microbiol* 2003, **11**:30–6.

7. Al-Fattani MA, Douglas LJ: **Penetration of Candida biofilms by antifungal agents.** *Antimicrob Agents Chemother* 2004, **48**:3291–7.

8. Xiao H, Ye X, Liu Q, Li L: **Antibiotic susceptibility and genotype patterns of Pseudomonas aeruginosa from mechanical ventilation-associated pneumonia in intensive care units.** *Biomed reports* 2013, **1**:589–593.

9. Hengzhuang W, Wu H, Ciofu O, Song Z, Høiby N: **Pharmacokinetics/pharmacodynamics of colistin and imipenem on mucoid and nonmucoid Pseudomonas aeruginosa biofilms.** *Antimicrob Agents Chemother* 2011, **55**:4469–74.

10. Uppuluri P, Srinivasan A, Ramasubramanian A, Lopez-Ribot JL: **Effects of fluconazole, amphotericin B, and caspofungin on Candida albicans biofilms under conditions of flow and on biofilm dispersion.** *Antimicrob Agents Chemother* 2011, **55**:3591–3.

11. Ramage G, VandeWalle K, Bachmann SP, Wickes BL, López-Ribot JL: **In vitro pharmacodynamic properties of three antifungal agents against preformed Candida albicans biofilms determined by time-kill studies.** *Antimicrob Agents Chemother* 2002, **46**:3634–6.

12. Roberts JA, Norris R, Paterson DL, Martin JH: **Therapeutic drug monitoring of antimicrobials.** *Br J Clin Pharmacol* 2012, **73**:27–36.

13. Skindersoe ME, Alhede M, Phipps R, Yang L, Jensen PO, Rasmussen TB, Bjarnsholt T, Tolker-Nielsen T, Høiby N, Givskov M: **Effects of antibiotics on quorum sensing in Pseudomonas aeruginosa.** *Antimicrob Agents Chemother* 2008, **52**:3648–63.

14. D'Auria FD, Tecca M, Strippoli V, Salvatore G, Battinelli L, Mazzanti G: **Antifungal activity of Lavandula angustifolia essential oil against Candida albicans yeast and mycelial form.** *Med Mycol* 2005, **43**:391–6.

15. Hassan Abdel-Rhman S, Mostafa El-Mahdy A, El-Mowafy M, Hassan Abdel-Rhman S, Mostafa El-Mahdy A, El-Mowafy M: **Effect of Tyrosol and Farnesol on Virulence and Antibiotic Resistance of Clinical Isolates of *Pseudomonas aeruginosa***. *Biomed Res Int* 2015, **2015**:1–7.

16. Tetz G V, Artemenko NK, Tetz V V: **Effect of DNase and antibiotics on biofilm characteristics.** *Antimicrob Agents Chemother* 2009, **53**:1204–9.

17. Cotton LA, Graham RJ, Lee RJ: **The Role of Alginate in P. aeruginosa PAO1 Biofilm Structural Resistance to Gentamicin and Ciprofloxacin**. *J Exp Microbiol Immunol* , **13**:58–62.

18. Craigen B, Dashiff A, Kadouri DE: **The Use of Commercially Available Alpha-Amylase Compounds to Inhibit and Remove Staphylococcus aureus Biofilms.** *Open Microbiol J* 2011, **5**:21–31.

19. Alipour M, Suntres ZE, Omri A: **Importance of DNase and alginate lyase for enhancing free and liposome encapsulated aminoglycoside activity against Pseudomonas aeruginosa.** *J Antimicrob Chemother* 2009, **64**:317–25.

20. Roux D, Gaudry S, Dreyfuss D, El-Benna J, de Prost N, Denamur E, Saumon G, Ricard J-D: **Candida albicans impairs macrophage function and facilitates Pseudomonas aeruginosa pneumonia in rat.** *Crit Care Med* 2009, **37**:1062–7.

21. Howe RA, Spencer RC: **Macrolides for the treatment of Pseudomonas aeruginosa infections?** *J Antimicrob Chemother* 1997, **40**:153–5.

22. Nalca Y, Jänsch L, Bredenbruch F, Geffers R, Buer J, Häussler S: **Quorum-sensing antagonistic activities of azithromycin in Pseudomonas aeruginosa PAO1: a global approach.** *Antimicrob Agents Chemother* 2006, **50**:1680–8.

23. Sheng T, Ku N, Palanisamy SKA, Lee SA: **Susceptibility of Candida albicans biofilms to azithromycin, tigecycline and vancomycin and the interaction between tigecycline and antifungals**. *Int J Antimicrob Agents* 2010, **36**:441–446.

24. Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, Dussault P, Nickerson KW: **Quorum sensing in the dimorphic fungus Candida albicans is mediated by farnesol.** *Appl Environ Microbiol* 2001, **67**:2982–92.

25. Ramage G, Saville SP, Wickes BL, López-Ribot JL: **Inhibition of Candida albicans biofilm formation by farnesol, a quorum-sensing molecule.** *Appl Environ Microbiol* 2002, **68**:5459–63.

26. Brehm-Stecher BF, Johnson EA: **Sensitization of Staphylococcus aureus and Escherichia coli to antibiotics by the sesquiterpenoids nerolidol, farnesol, bisabolol, and apritone.** *Antimicrob Agents Chemother* 2003, **47**:3357–60.

27. Jabra-Rizk MA, Meiller TF, James CE, Shirtliff ME: **Effect of farnesol on Staphylococcus aureus biofilm formation and antimicrobial susceptibility.** *Antimicrob Agents Chemother* 2006, **50**:1463–9.

28. Semighini CP, Hornby JM, Dumitru R, Nickerson KW, Harris SD: **Farnesol-induced apoptosis in Aspergillus nidulans reveals a possible mechanism for antagonistic interactions between fungi.** *Mol Microbiol* 2006, **59**:753–64.

29. Yang L, Rybtke MT, Jakobsen TH, Hentzer M, Bjarnsholt T, Givskov M, Tolker-Nielsen T: **Computer-aided identification of recognized drugs as Pseudomonas aeruginosa quorum-sensing inhibitors.** *Antimicrob Agents Chemother* 2009, **53**:2432–43.

30. Alviano WS, Mendonça-Filho RR, Alviano DS, Bizzo HR, Souto-Padrón T, Rodrigues ML, Bolognese AM, Alviano CS, Souza MMG: **Antimicrobial activity of Croton cajucara Benth linalool-rich essential oil on artificial biofilms and planktonic microorganisms.** *Oral Microbiol Immunol* 2005, **20**:101–5.

31. Zore GB, Thakre AD, Jadhav S, Karuppayil SM: **Terpenoids inhibit Candida albicans growth by affecting membrane integrity and arrest of cell cycle.** *Phytomedicine* 2011, **18**:1181–90.

32. Hsu C-C, Lai W-L, Chuang K-C, Lee M-H, Tsai Y-C: **The inhibitory activity of linalool against the filamentous growth and biofilm formation in Candida albicans.** *Med Mycol* 2013, **51**:473–82.

33. Berditsch M, Jäger T, Strempel N, Schwartz T, Overhage J, Ulrich AS: **Synergistic effect of membrane-active peptides polymyxin B and gramicidin S on multidrug-resistant strains and biofilms of Pseudomonas aeruginosa.** *Antimicrob Agents Chemother* 2015, **59**:5288–96.

34. Odds FC: **Synergy, antagonism, and what the chequerboard puts between them.** *J Antimicrob Chemother* 2003, **52**:1.

35. Landman D, Bratu S, Alam M, Quale J: **Citywide emergence of Pseudomonas aeruginosa strains with reduced susceptibility to polymyxin B.** *J Antimicrob Chemother* 2005, **55**:954–7.

36. RICHARDS RME, XING DKL: **Investigation of Synergism between Combinations of Ciprofloxacin, Polymyxin, Sulphadiazine and p-Aminobenzoic acid**. *J Pharm Pharmacol* 1993, **45**:171–175.

37. Wei Q, Ma LZ: **Biofilm matrix and its regulation in Pseudomonas aeruginosa.** *Int J Mol Sci* 2013, **14**:20983–1005.

38. Davey HM, Kell DB: **Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses.** *Microbiol Rev* 1996, **60**:641–96.

39. Lu Y, Su C, Wang A, Liu H: **Hyphal Development in Candida albicans Requires Two Temporally Linked Changes in Promoter Chromatin for Initiation and Maintenance**. *PLoS Biol* 2011, **9**:e1001105.

40. Ciandrini E, Campana R, Federici S, Manti A, Battistelli M, Falcieri E, Papa S, Baffone W: **In vitro activity of Carvacrol against titanium-adherent oral biofilms and planktonic cultures**. *Clin Oral Investig* 2014, **18**:2001–2013.

41. Oliveira F, Lima CA, Brás S, França Â, Cerca N: **Evidence for inter- and intraspecies biofilm formation variability among a small group of coagulase-negative staphylococci**. *FEMS Microbiol Lett* 2015, **362**.

42. Camps M, Barani A, Gregori G, Bouchez A, Le Berre B, Bressy C, Blache Y, Briand J-F: **Antifouling coatings influence both abundance and community structure of colonizing biofilms: a case study in the Northwestern Mediterranean Sea.** *Appl Environ Microbiol* 2014, **80**:4821–31.

43. Dashper SG, Liu S-W, Walsh KA, Adams GG, Stanton DP, Ward BR, Shen P, O'Brien-Simpson NM, Reynolds EC: **Streptococcus mutans biofilm disruption by κ-casein glycopeptide**. *J Dent* 2013, **41**:521–527.

44. Van Acker H, Sass A, Bazzini S, De Roy K, Udine C, Messiaen T, Riccardi G, Boon N, Nelis HJ, Mahenthiralingam E, Coenye T, Vanlaere E, Baldwin A, Gevers D, Henry D, Brandt E De, LiPuma J, Jones A, Dodd M, Govan J, Barcus V, Doherty C, Soyza A De, Morris K, McDowell A, Doherty C, Archer L, Mahenthiralingam E, Urban T, Goldberg J, et al.: **Biofilm-Grown Burkholderia cepacia Complex Cells Survive Antibiotic Treatment by Avoiding Production of Reactive Oxygen Species**. *PLoS One* 2013, **8**:e58943.

45. Bustin SA, Benes V, Nolan T, Pfaffl MW: **Quantitative real-time RT-PCR–a perspective.** *J Mol Endocrinol* 2005, **34**:597–601.

46. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT: **The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments**. *Clin Chem* 2009, **55**.

Chapter 4

CONCLUDIND REMARKS AND FUTURE PERPECTIVES

CONCLUDING REMARKS

Understand the behavior of pathogens when they are in polymicrobial consortia, is an important step in clinical context to achieve an effectiveness treatment in case of disease. In this scope, the study of inter-kingdom communication in dual-species biofilms is the extreme importance in context of VAP infection. Under the experimental conditions, microorganisms had a different answer to the same antimicrobials when are alone or in combination. Different antimicrobials were tested and were found that some can inhibit biofilm formation and others were ineffective to any condition studied. Patulin, alginate lyase and desoxirribonuclease had no effect against single- and mixed-species biofilms of *P. aeruginosa* and *C. albicans*. On the other hand, chlorogenic acid, farnesol and linalool three compounds of natural origin show effect against pre-established biofilms. Linalool was the most effective natural compound and affected in particular *C. albicans* in single and mixed biofilms. Salicylic acid and AZT present slightly reduction in cells of *C. albicans* and *P. aeruginosa* respectively and CIP, another commercial drug, was the most effective QSI tested showing reductions for both pathogens in single and mixed-species consortia. Some combinations of these antimicrobials show that may be useful to obtain higher reductions in biofilms-encased cells. Farnesol/ CIP and linalool/ CIP were effective against single biofilms and presented significant reduction in mixed biofilms (in particular for *C. albicans*). These antimicrobials have part of new therapies that had emerged due to failure of conventional antimicrobials (antibiotics and antifungals) to treat VAP patients.

FUTURE PERPECTIVES

Because is important to understand the mechanisms underlying the changes that occur during co-infection, bacterial transcriptome during pathogen-pathogen interactions is a fundamental step to understand the infectious processes caused by human pathogens. In this sense, there is already a vast work done until now that will allow to have the following work simplified. In future work, the goal is to repeat the extractions to obtain the intended transcriptomic analysis by RNA-seq. In this sense is necessary workaround the arrival time to the enterprise chosen for making this analysis. Another hypothesis will make the construction of libraries and then send to external company.

The use of more robust techniques as RNA-seq and flow cytometry require a lot of optimization to achieve credible and reliable results. Hence all decisions to be taken and the results obtained have to be well analyzed. In this moment, there is a challenging work ahead but it is part of the goals

stick to it. In the future, it is also intended to continue the work of combinations of QSI to find better results against polymicrobial biofilms and continue the analysis of these biofilms by flow cytometry.

ANNEX I

RESULTS OF RNA QUALITY PROVIDED BY THE COMPANY

BGI Sample Testing Report

1. Project Information

Report No.: THKe16042102

Project Name	library sequencing for Dismed			**Project No.**	F15FTSEUHT1142		
Customer Name	Analia Louren?o			**Customer Unit**	Dismed SA		
Lab Sample Collector	wanpakkiu			**Lab Sample Receiving Date**	20160421		
Lab Sample Tester	Tang Kam Yu, Wan Pak Kiu, Yeung Cheuk Yiu, Ho Ching Yu			**Lab Sample Testing Date**	20160421		
Lab Name	BGI-Hong Kong NGS Lab			**Lab Address**	16 Dai Fu Street, Tai Po Industrial Estate, Tai Po, New Territories, Hong Kong.		
Reported by	Ho Ching Yu	**Inspected by**	tangkamyu	**Approved by**	YEUNG Cheuk Yiu	**Report Date**	20160422

2. Sample Test Method

①Method of concentration determination: □ QubitFluorometer, ■ Agilent 2100, □ NanoDrop, □MicroplateReader;
②Method of OD260/280 & OD260/230 test: ■ NanoDrop;
③Method of 28S/18S & 23S/16S test: ■ Agilent 2100;
④Method of RIN test: ■ Agilent 2100;

3. Sample Test Result

No.	Sample Name	Sample Number	Tube No.	Concen-tration (ng/μL)	Volume (μL)	Total Mass (μg)	OD260/280	OD260/230	RIN	28S/ 18S	Library Type	Test Result	Remark
1	CA	8521604002545	1	140	46	6.44	2.09	2.13	5.5	1.1	HiSeq Transcriptome	Level D	RIN＜6.5.The baseline is not smooth.
2	PA	8521604002546	1	320	41	13.12	1.96	2.20	7.5	1.7	HiSeq Transcriptome	Level A	
3	CP	8521604002547	1	183	80	14.64	2.02	1.69	4.5	1.5	HiSeq Transcriptome	Level D	RIN＜5.0.
4	CP1	8521604002548	1	610	49	29.89	2.00	2.01	5.7	1.9	HiSeq Transcriptome	Level C	RIN＜6.5.
5	CP2	8521604002549	1	854	38	32.45	1.94	1.55	9.6	1.1	HiSeq Transcriptome	Level A	
6	P	8521604002550	1	1072	31	33.23	2.08	2.37	9.3	1.5	HiSeq Transcriptome	Level A	
7	B	8521604002551	1	970	31	30.07	2.03	2.36	9.3	1.5	HiSeq Transcriptome	Level A	
8	BRC	8521604002552	1	875	36	31.5	2.07	2.38	9.7	1.1	HiSeq Transcriptome	Level A	
9	SA1	8521604002553	1	203	13	2.64	2.07	1.89	8.2	0.6	HiSeq Transcriptome	Level D	23S/16S＜0.8.
10	SA2	8521604002554	1	84	13	1.09	2.10	2.01	8.4	0.8	HiSeq Transcriptome	Level C	23S/16S＜1.0.
11	SA3	8521604002555	1	335	14	4.69	2.15	1.40	8.5	1.5	HiSeq Transcriptome	Level A	

Note*

1.The 260/280 and 260/230 absorbance ratio <1.8:It may lead to library construction failure, library production too low to sequence or insufficient sequencing data quantity; and it may affect randomicity and cause bias. The closer to the standard the smaller risk ,conversely the bigger.

2.The test result based on the《RNA sequencing sample quality standards》 explains whether the testing sample meets the requirement of library construction.

a） Level A means the sample is qualified, and the amount of sample satisfies two times library construction or more.

b） Level B means the sample is qualified, but the amount of sample only satisfies one time library construction.

c） Level C means the sample does not totally meet the requirements of library construction and sequencing. BGI can try to construct library but sequencing quality is not guaranteed.

d） Level D means the sample does not meet the requirements of library construction and sequencing. BGI does not suggest using the sample.

3.For samples of Level A&B, the first success rate of Low-input (200ng) RNA (Quantification)library construction is above 95%, based on experiment statistics in BGI.

4.For samples of Level C&D, Low-input (200ng) RNA (Quantification) library construction has the following risk at least, based on experiment statistics in BGI.

a) Deficient or too Low Quantity of RNA: It may lead to library construction failure, too low library production to sequence or insufficient sequencing data quantity; and it may affect data randomness and cause bias.

b) Sample Degradation: It may lead to library construction failure; may lead to high proportion of duplication and poor randomness of sequencing data.

c) Pollution by Protein or Insoluble Impurity: It may affect normal electrophoretic separation, result in incorrectness of cutting gel, and affect library quality. It also may affect the efficiency of mRNA isolation using magnetic bead and reverse transcription and then lead to library construction failure. Even library is carried out sequencing; it may lead to poor randomness of library, high proportion of duplication and inaccurate gene expression quantification (common in plant sample containing much saccharides and phenols).

d) Remains of rRNA in rRNA depleted Samples: The remains may lead to too high ratio of rRNA in sequencing data.

e) mRNA Samples: RNA Integrity is hard to test, Quality cannot be guaranteed.

f) 5S peak on the high side will affect the quantitative veracity in samples testing and conduce to the downstream inaccuracy and the poor data.

g)For plant,fungi and bacteria,the 260/280 and 260/230 absorbance ratio <1.8,It may have Impurity,it may inhibit enzymatic reaction and affects the successful rate of library construction and insufficient sequencing data quantity.This kind of samples can have 90% successful rate in library construction.

5.If COs insist on constructing library with samples in Level C&D, COs shall take the responsibility and risk involved in this matter.
6.The following conditions carry moderate risk, and we can try to construct libraries with them:
a) The RIN value is slightly under standard, but basic line is smooth.
b) The RIN value reaches standard, but basic line is slightly rise.
c) The basic line is smooth and RIN value reaches standard, but 5S peak is slightly high.
d) 28S/18S or 23S/16S value is slightly under standard, but the basic line is smooth.
e) Generally qualified, but total amount is below Level B.
f) For plant samples, the OD 260/280, 260/230 is slightly under standard, and samples status is not ropy.
g) For soil bacterial and ocean microorganism samples are not able for Meta Strand-Specific Transcriptome library construction.

4. Appendix

Appendix 1: Test results of NanoDrop
Appendix 2: Test results of Agilent 2100
Appendix 3: Original information of sample

5. Statement

1. The results shown in this report refer only to the sample of the report unless otherwise stated.

2. This test report cannot be copied partly without the prior written permission of the Lab.

Appendix 1: Test results of NanoDrop

1. Pre-treatment

After the sample melted the ice, centrifuged and fully mixed, take appropriate samples for testing.

2. Test Result

Sample Name	Blanking for test	OD260/280	OD260/230	Remark
CA	DEPC treated water	2.09	2.13	
PA	DEPC treated water	1.96	2.20	
CP	DEPC treated water	2.02	1.69	
CP1	DEPC treated water	2.00	2.01	
CP2	DEPC treated water	1.94	1.55	
P	DEPC treated water	2.08	2.37	
B	DEPC treated water	2.03	2.36	
BRC	DEPC treated water	2.07	2.38	
SA1	DEPC treated water	2.07	1.89	
SA2	DEPC treated water	2.10	2.01	
SA3	DEPC treated water	2.15	1.40	

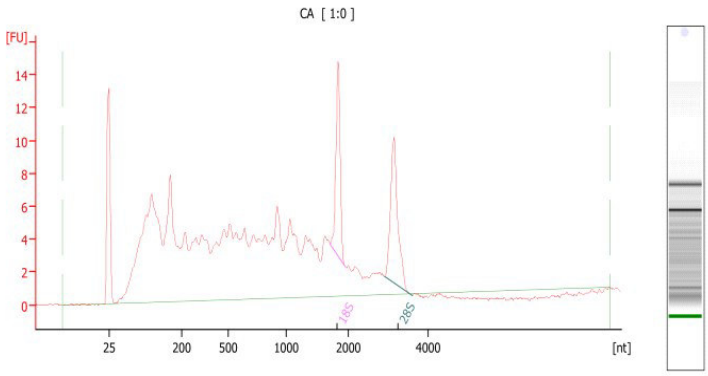
Appendix 2: Test results of Agilent 2100

1. Pre-treatment

After the sample melted the ice, centrifuged and fully mixed, take appropriate samples for testing.

2. Test Result

(1) Sample name: CA



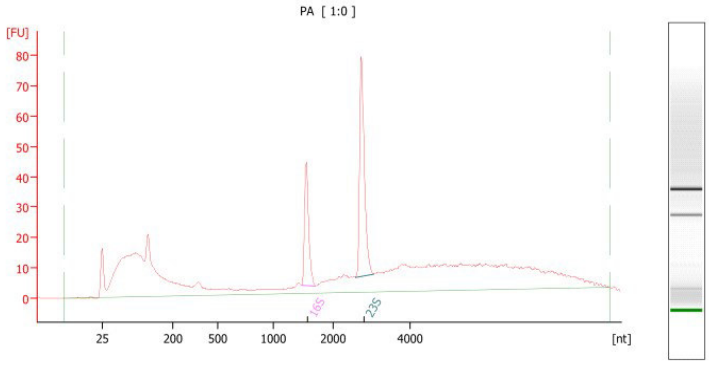
Overall Results for sample 1 : CA

RNA Area:	252.1	RNA Integrity Number (RIN):	5.5 (B.02.07)
RNA Concentration:	140 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	1.1	Result Flagging Label:	RIN: 5.50

Fragment table for sample 1 : CA

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,708	1,949	12.3	4.9
28S	2,920	3,613	13.3	5.3

(2) Sample name: PA



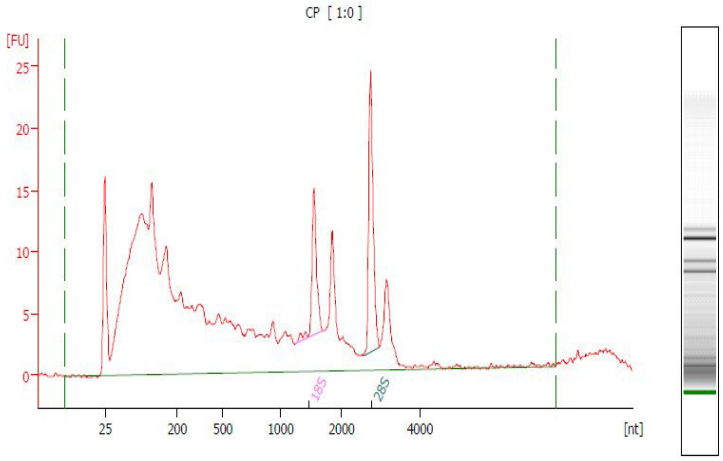
Overall Results for sample 1 : PA

RNA Area:	731.8	RNA Integrity Number (RIN):	7.5 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	320 ng/µl		
rRNA Ratio [23s / 16s]:	1.7		

Fragment table for sample 1 : PA

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,468	1,698	43.0	5.9
23S	2,594	3,054	73.6	10.1

(3) Sample name: CP



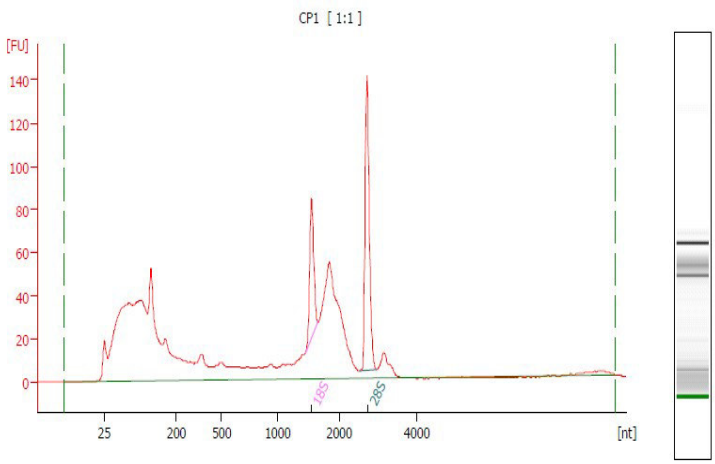
Overall Results for sample 2 : CP

RNA Area:	418.0	RNA Integrity Number (RIN):	4.5 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	183 ng/µl		
rRNA Ratio [28s / 18s]:	1.5		

Fragment table for sample 2 : CP

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,261	1,691	13.6	3.3
28S	2,578	2,966	20.9	5.0

(4) Sample name: CP1



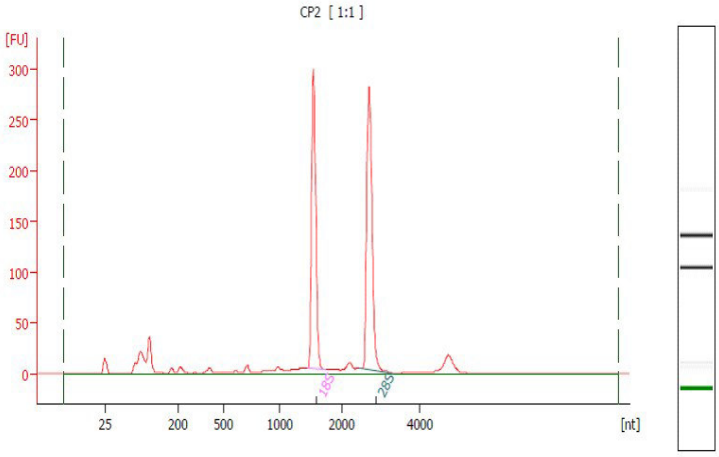
Overall Results for sample 3 : CP1

RNA Area:	1,397.7	RNA Integrity Number (RIN):	5.7 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	305 ng/µl		
rRNA Ratio [28s / 18s]:	1.9		

Fragment table for sample 3 : CP1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,446	1,647	64.4	4.6
28S	2,521	2,964	122.1	8.7

(5) Sample name: CP2



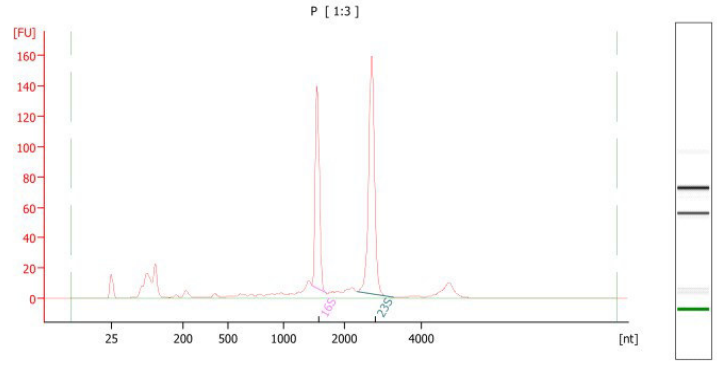
Overall Results for sample 4 : CP2

RNA Area:	975.6	RNA Integrity Number (RIN):	9.6 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	427 ng/µl		
rRNA Ratio [28s / 18s]:	1.1		

Fragment table for sample 4 : CP2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,449	1,731	302.3	31.0
28S	2,479	3,307	322.5	33.1

(6) Sample name: P



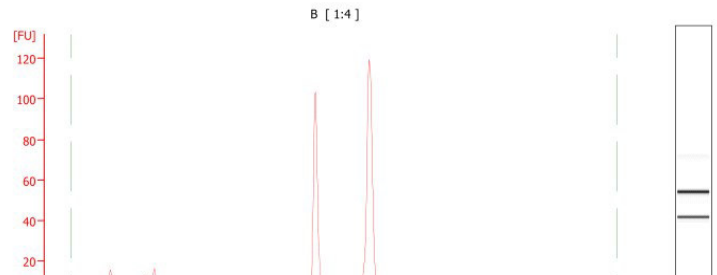
Overall Results for sample 5 : P

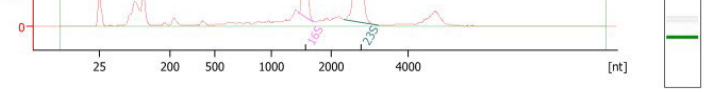
RNA Area:	613.7	RNA Integrity Number (RIN):	9.3 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	268 ng/µl		
rRNA Ratio [23s / 16s]:	1.5		

Fragment table for sample 5 : P

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,458	1,695	135.0	22.0
23S	2,337	3,264	202.0	32.9

(7) Sample name: B





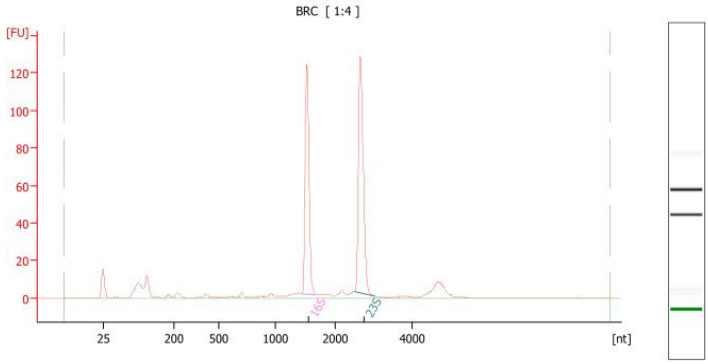
Overall Results for sample 6 : B

RNA Area:	443.5	RNA Integrity Number (RIN):	9.3 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	194 ng/µl		
rRNA Ratio [23s / 16s]:	1.5		

Fragment table for sample 6 : B

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,472	1,701	99.3	22.4
23S	2,336	3,224	146.2	33.0

(8) Sample name: BRC



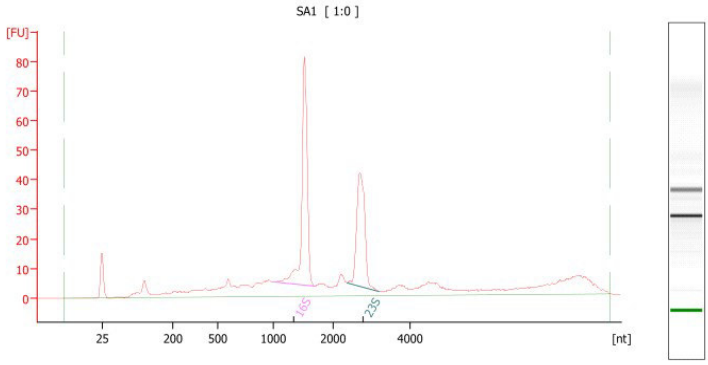
Overall Results for sample 7 : BRC

RNA Area:	400.0	RNA Integrity Number (RIN):	9.7 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	175 ng/µl		
rRNA Ratio [23s / 16s]:	1.1		

Fragment table for sample 7 : BRC

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,445	1,683	121.1	30.3
23S	2,493	3,038	135.0	33.7

(9) Sample name: SA1



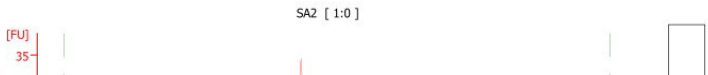
Overall Results for sample 8 : SA1

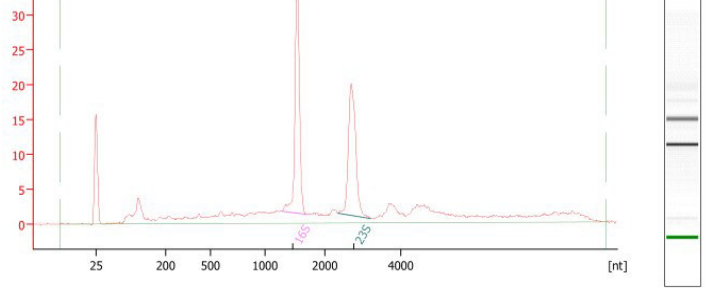
RNA Area:	463.5	RNA Integrity Number (RIN):	8.2 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	203 ng/µl		
rRNA Ratio [23s / 16s]:	0.6		

Fragment table for sample 8 : SA1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,008	1,698	109.3	23.6
23S	2,363	3,213	69.4	15.0

(10) Sample name: SA2





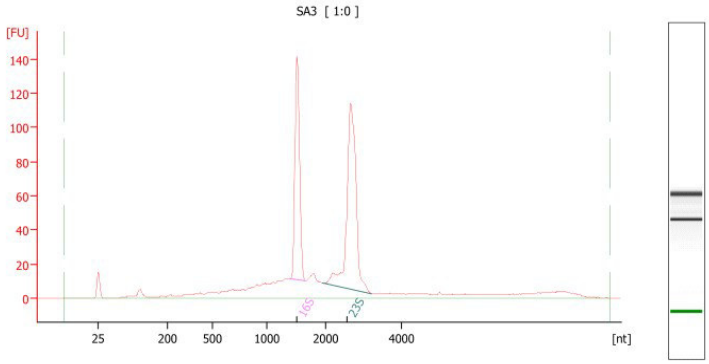
Overall Results for sample 9 : SA2

RNA Area:	192.3	RNA Integrity Number (RIN):	8.4 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	84 ng/µl		
rRNA Ratio [23s / 16s]:	0.8		

Fragment table for sample 9 : SA2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,266	1,683	39.8	20.7
23S	2,347	3,204	32.7	17.0

(11) Sample name: SA3



Overall Results for sample 10 : SA3

RNA Area:	766.5	RNA Integrity Number (RIN):	8.5 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	335 ng/µl		
rRNA Ratio [23s / 16s]:	1.5		

Fragment table for sample 10 : SA3

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,389	1,660	153.4	20.0
23S	1,940	3,208	235.7	30.7

Sample Name	Test Instrument	Test Kit	Dilution Ratio(×)	Test Concentration (ng/µL)	Concentration of original sample(ng/µL)	RIN	28S/ 18S	Remark
CA	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	140	140	5.5	1.1	
PA	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	320	320	7.5	1.7	
CP	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	183	183	4.5	1.5	
CP1	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	305	610	5.7	1.9	
CP2	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	427	854	9.6	1.1	
P	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	4	268	1072	9.3	1.5	
B	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	5	194	970	9.3	1.5	
BRC	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	5	175	875	9.7	1.1	
SA1	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	203	203	8.2	0.6	
SA2	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	84	84	8.4	0.8	
SA3	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	335	335	8.5	1.5	

Appendix3: Original information of sample

Sample Type:
total RNA
Sample status:
溶于无RNase水(或DEPC水)
Further Information:

Sample Name	Species	No. of Tubes	Concentration (ng/μL)	Volume (μL)	Total Quantity (μg)	28S/18S	OD260/280	OD260/230	Remark
CA		1			0		0	0	西班牙
PA		1			0		0	0	西班牙
CP		1			0		0	0	西班牙
CP1		1			0		0	0	西班牙
CP2		1			0		0	0	西班牙
P		1			0		0	0	西班牙
B		1			0		0	0	西班牙
BRC		1			0		0	0	西班牙
SA1		1			0		0	0	西班牙
SA2		1			0		0	0	西班牙
SA3		1			0		0	0	西班牙

End of Report

BGI Sample Testing Report

1. Project Information

Report No.: THKe16052702

Project Name	20160420 RNA-Seq for Dismed			**Project No.**	F16FTSEUHT0362		
Customer Name	Analia Lourenco			**Customer Unit**	Dismed SA		
Lab Sample Collector	wanpakkiu			**Lab Sample Receiving Date**	20160525		
Lab Sample Tester	Tang Kam Yu, Wan Pak Kiu, Tsang Kwun Wai. Ho Ching Yu, Wong Kwun Kiu			**Lab Sample Testing Date**	20160526		
Reported by	Wong Kwun Kiu	**Inspected by**	tangkamyu	**Approved by**	Tsang Kwun Wai	**Report Date**	20160528

2. Sample Test Method

①Method of concentration determination: □ QubitFluorometer, ■ Agilent 2100, □ NanoDrop, □MicroplateReader;
②Method of 28S/18S & 23S/16S test: ■ Agilent 2100;
③Method of RIN test: ■ Agilent 2100;

3. Sample Test Result

No.	Sample Name	Sample Number	Tube No.	Concen-tration (ng/μL)	Volume (μL)	Total Mass(μg)	RIN	23S/ 16S	Library Type	Test Result	Remark
1	C1	8521605004427	1	41	14	0.57	3.0	0.6	HiSeq Transcriptome	Level D	RIN＜5.0.
2	C2	8521605004428	1	68	21	1.43	2.6	0.1	HiSeq Transcriptome	Level D	RIN＜5.0.
3	C3	8521605004429	1	14	21	0.29	2.5	0.0	HiSeq Transcriptome	Level D	RIN＜5.0.
4	M1	8521605004430	1	84	15	1.26	3.5	0.8	HiSeq Transcriptome	Level D	RIN＜6.0.
5	M2	8521605004431	1	36	20	0.72	4.4	0.4	HiSeq Transcriptome	Level D	RIN＜6.0.
6	M3	8521605004432	1	90	20	1.8	2.8	1.6	HiSeq Transcriptome	Level D	RIN＜6.0.
7	M4	8521605004433	1	100	8	0.8	4.1	1.3	HiSeq Transcriptome	Level D	RIN＜6.0.
8	M5	8521605004434	1	92	7	0.64	5.0	0.7	HiSeq Transcriptome	Level D	RIN＜6.0.
9	M6	8521605004435	1	62	12	0.74	4.5	1.4	HiSeq Transcriptome	Level D	RIN＜6.0.
10	M7	8521605004436	1	38	8	0.3	4.6	1.3	HiSeq Transcriptome	Level D	RIN＜6.0.
11	M8	8521605004437	1	266	19	5.05	3.5	0.3	HiSeq Transcriptome	Level D	RIN＜6.0.
12	M9	8521605004438	1	202	25	5.05	4.1	1.8	HiSeq Transcriptome	Level D	RIN＜6.0.
13	M10	8521605004439	1	472	20	9.44	4.3	2.1	HiSeq Transcriptome	Level D	RIN＜6.0.
14	T1	8521605004440	1	130	11	1.43	6.6	1.8	HiSeq Transcriptome	Level C	RIN＜7.0.
15	T2	8521605004441	1	84	11	0.92	5.9	1.9	HiSeq Transcriptome	Level D	RIN＜6.0.
16	T3	8521605004442	1	220	9	1.98	7.2	1.4	HiSeq Transcriptome	Level B	
17	T4	8521605004443	1	332	9	2.99	7.2	1.5	HiSeq Transcriptome	Level A	
18	T5	8521605004444	1	422	11	4.64	7.4	0.0	HiSeq Transcriptome	Level D	23S/16S＜0.8.
19	T6	8521605004445	1	300	10	3	7.4	0.0	HiSeq Transcriptome	Level D	23S/16S＜0.8.
20	T7	8521605004446	1	734	10	7.34	6.4	1.4	HiSeq Transcriptome	Level C	RIN＜6.0.
21	T8	8521605004447	1	638	16	10.21	5.7	0.0	HiSeq Transcriptome	Level D	RIN＜6.0.
22	SA4	8521605004448	1	84	14	1.18	7.4	0.0	HiSeq Transcriptome	Level D	23S/16S＜0.8.
23	SA5	8521605004449	1	122	12	1.46	7.7	0.4	HiSeq Transcriptome	Level D	23S/16S＜0.8.

Note*

1.The test result based on the《RNA sequencing sample quality standards》 explains whether the testing sample meets the requirement of library construction.
a) Level A means the sample is qualified, and the amount of sample satisfies two times library construction or more.
b) Level B means the sample is qualified, but the amount of sample only satisfies one time library construction.
c) Level C means the sample does not totally meet the requirements of library construction and sequencing. BGI can try to construct library but sequencing quality is not guaranteed.
d) Level D means the sample does not meet the requirements of library construction and sequencing. BGI does not suggest using the sample.
2.For samples of Level A&B, the first success rate of Transcriptome Library construction is above 95%, based on historical statistics in BGI.
3.For samples of Level C&D, Transcriptome Library construction has the following risk at least, based on historical statistics in BGI.:
a) Deficient or too Low Mass RNA: It may lead to library construction failure, too low library production to sequence or

insufficient sequencing data amount; and it may affect data randomness and cause bias.
b) Sample Degradation: It may lead to library construction failure; may lead to high proportion of duplication and poor randomness of sequencing data ;may lead to inaccurate gene expression quantitation.
d) Concentrationof RNA is too high , Sampling is inaccurate: It may lead to library construction failure.
c) 5S peak on the high side will affect the quantitative inaccuracy and conduce to the inaccuracy of loading amount and the poor data quality.
d)For plant,fungi and bacteria,the 260/280 and 260/230 absorbance ratio <1.8,It may have Impurity ,it may inhibit enzymatic reaction and affects the successful rate of library construction and insufficient sequencing data quantity. This kind of samples can have 90% successful rate in library construction.
4.If COs insist on constructing library with samples in Level C&D, COs shall take the responsibility and risk involved in this matter.
5.The following conditions carry moderate risk, and we can try to construct libraries with them:
a) The RIN value is slightly under standard, but basic line is smooth.
b) The RIN value reaches standard, but basic line is slightly rise.
c) The basic line is smooth and RIN value reaches standard, but 5S peak is slightly high.
d) 28S/18S or 23S/16S value is slightly under standard, but the basic line is smooth.
e) Generally qualified, but total amount is below Level B.
f) For plant samples, the OD 260/280, 260/230 is slightly under standard, and samples status is not ropy.
g) For soil bacterial and ocean microorganism samples are not able for Meta Strand-Specific Transcriptome library construction.

4. Appendix

Appendix 1: Test results of Agilent 2100
Appendix 2: Original information of sample

5. Statement

1. The results shown in this report refer only to the sample of the report unless otherwise stated.

2. This test report cannot be copied partly without the prior written permission of the Lab.

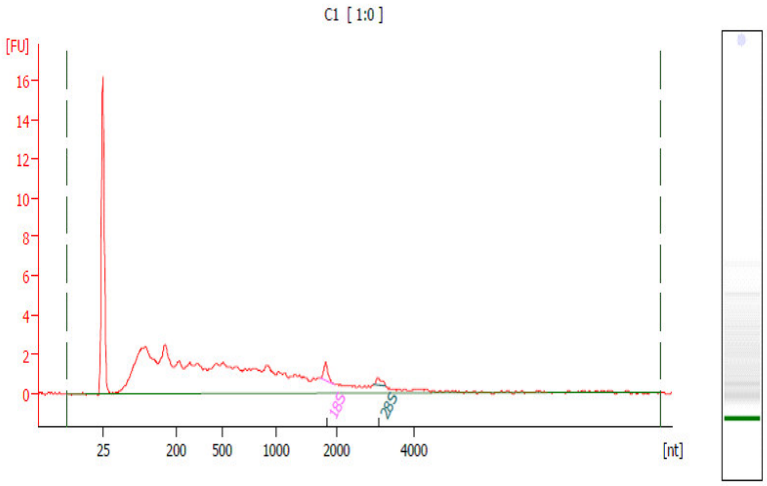
Appendix 1: Test results of Agilent 2100

1. Pre-treatment

After the sample melted the ice, centrifuged and fully mixed, take appropriate samples for testing.

2. Test Result

(1) Sample name: C1



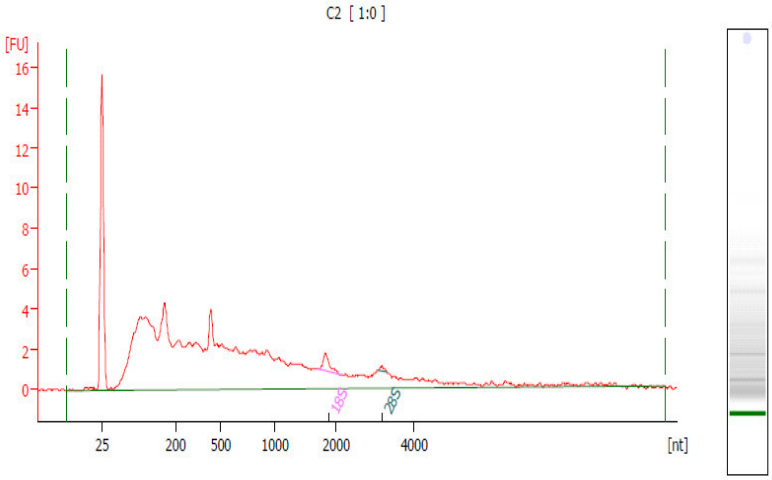
Overall Results for sample 10 : C1

RNA Area:	79.6	RNA Integrity Number (RIN):	3 (B.02.07)
RNA Concentration:	41 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.6	Result Flagging Label:	RIN:3

Fragment table for sample 10 : C1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,744	1,974	0.9	1.2
28S	2,962	3,285	0.5	0.6

(2) Sample name: C2



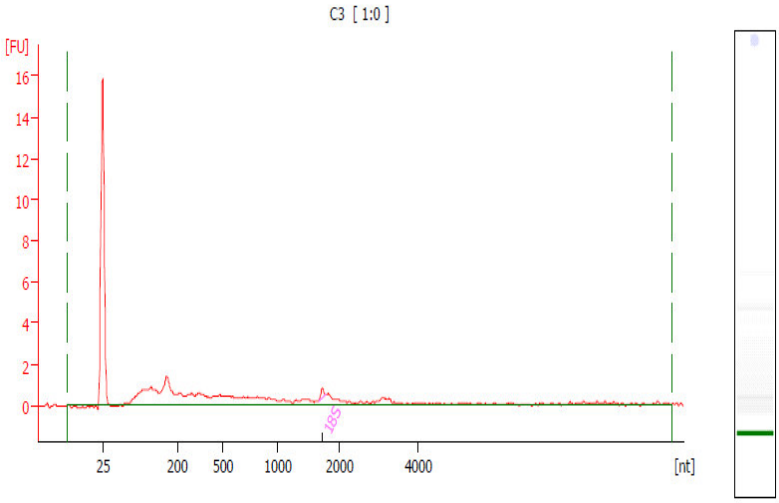
Overall Results for sample 11 : C2

RNA Area:	132.0	RNA Integrity Number (RIN):	2.6 (B.02.07)
RNA Concentration:	68 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.1	Result Flagging Label:	RIN: 2.60

Fragment table for sample 11 : C2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,711	2,142	1.4	1.1
28S	3,070	3,302	0.2	0.1

(3) Sample name: C3



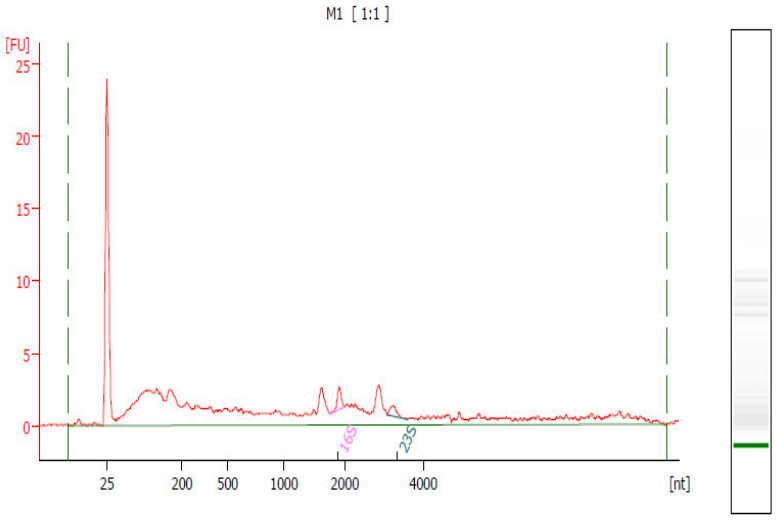
Overall Results for sample 12 : C3

RNA Area:	28.0	RNA Integrity Number (RIN):	2.5 (B.02.07)
RNA Concentration:	14 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.0	Result Flagging Label:	RIN: 2.50

Fragment table for sample 12 : C3

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,660	1,771	0.3	1.1

(4) Sample name: M1



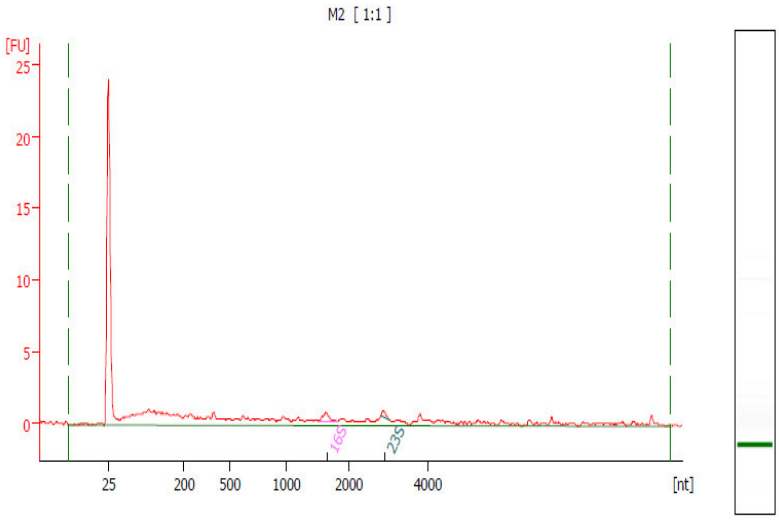
Overall Results for sample 1 : M1

RNA Area:	108.7	rRNA Ratio [23s / 16s]:	0.8
RNA Concentration:	42 ng/µl	RNA Integrity Number (RIN):	3.5 (B.02.08)

Fragment table for sample 1 : M1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,775	2,004	1.3	1.2
23S	3,084	3,609	1.1	1.0

(5) Sample name: M2



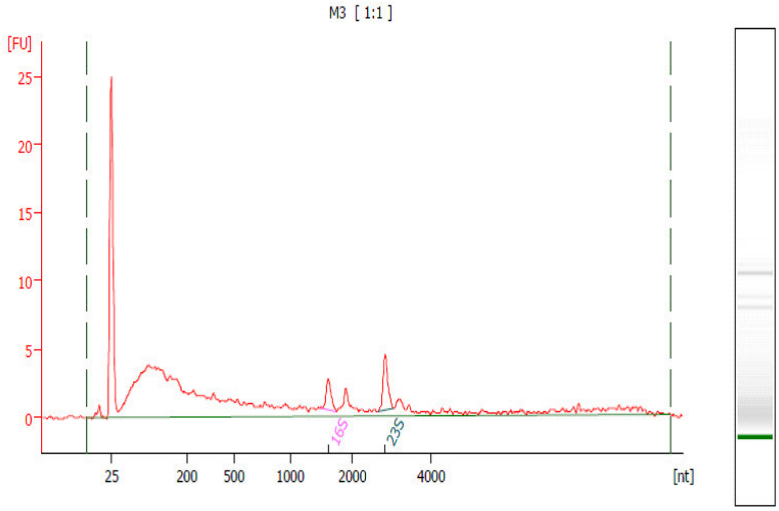
Overall Results for sample 2 : M2

RNA Area:	45.8	rRNA Ratio [23s / 16s]:	0.4
RNA Concentration:	18 ng/µl	RNA Integrity Number (RIN):	4.4 (B.02.08)

Fragment table for sample 2 : M2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,524	1,808	0.9	2.0
23S	2,807	3,053	0.4	0.8

(6) Sample name: M3



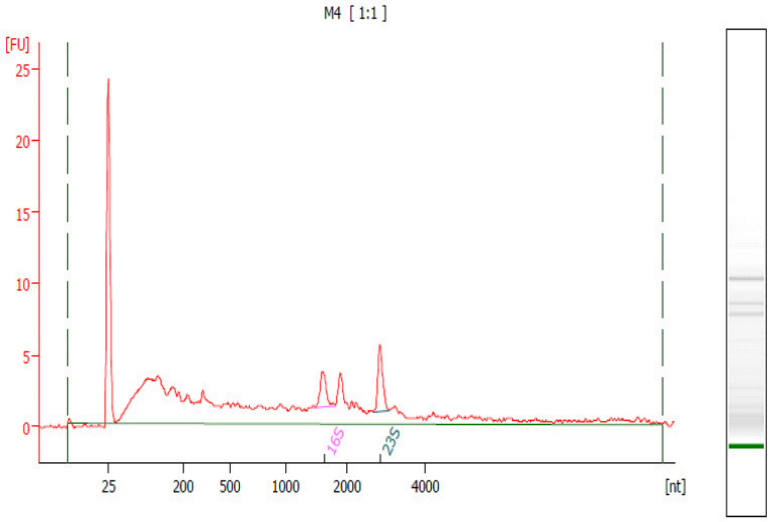
Overall Results for sample 3 : M3

RNA Area:	116.2	RNA Integrity Number (RIN):	2.8 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	45 ng/µl		
rRNA Ratio [23s / 16s]:	1.6		

Fragment table for sample 3 : M3

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,519	1,743	2.6	2.2
23S	2,669	3,052	4.2	3.6

(7) Sample name: M4



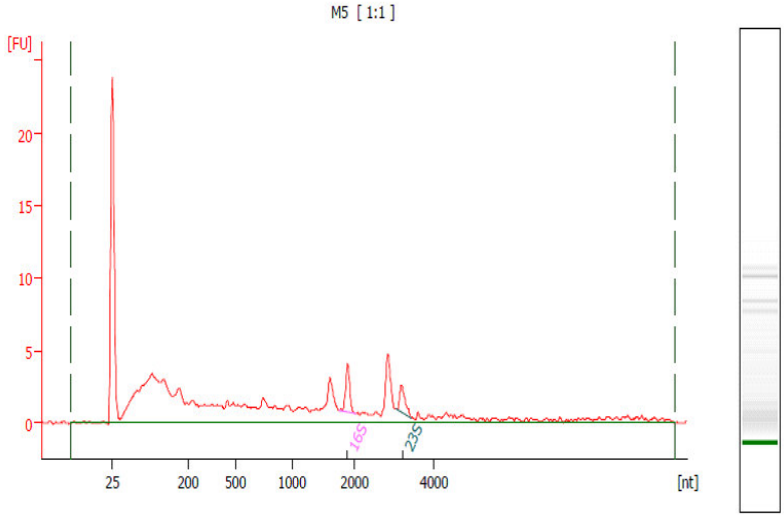
Overall Results for sample 4 : M4

RNA Area:	129.6	rRNA Ratio [23s / 16s]:	1.3
RNA Concentration:	50 ng/µl	RNA Integrity Number (RIN):	4.1 (B.02.08)

Fragment table for sample 4 : M4

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,489	1,812	3.7	2.8
23S	2,685	3,067	4.9	3.8

(8) Sample name: M5



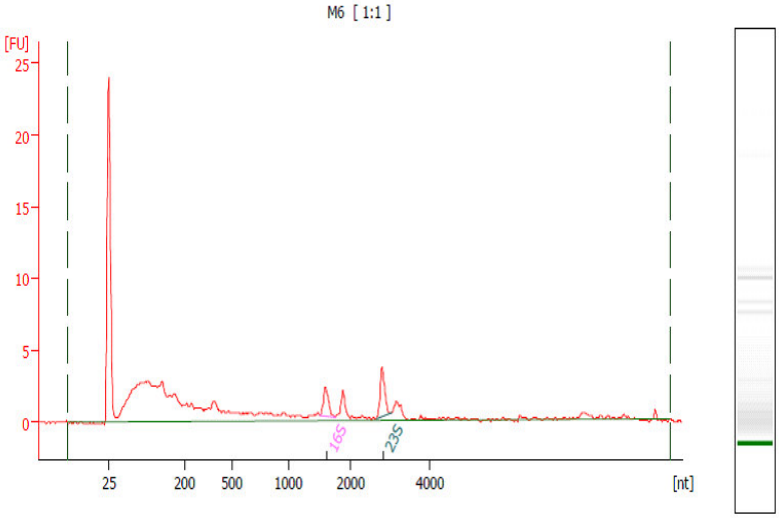
Overall Results for sample 5 : M5

RNA Area:	120.5	rRNA Ratio [23s / 16s]:	0.7
RNA Concentration:	46 ng/µl	RNA Integrity Number (RIN):	5 (B.02.08)

Fragment table for sample 5 : M5

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,763	2,027	3.0	2.5
23S	3,052	3,419	2.0	1.7

(9) Sample name: M6



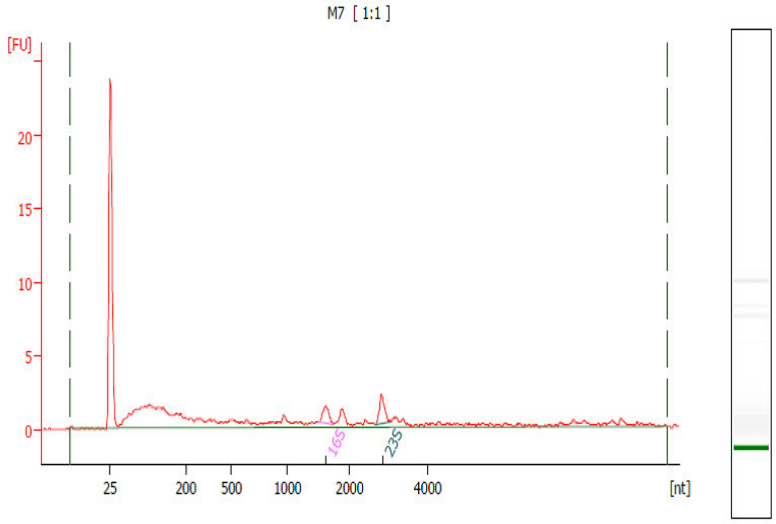
Overall Results for sample 6 : M6

RNA Area:	80.3	rRNA Ratio [23s / 16s]:	1.4
RNA Concentration:	31 ng/µl	RNA Integrity Number (RIN):	4.5 (B.02.08)

Fragment table for sample 6 : M6

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,519	1,753	2.5	3.1
23S	2,654	3,037	3.4	4.3

(10) Sample name: M7



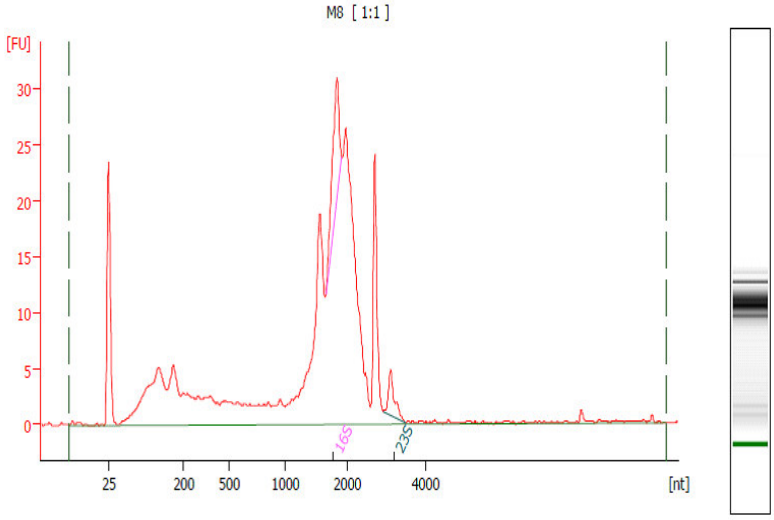
Overall Results for sample 7 : M7

RNA Area:	48.9	rRNA Ratio [23s / 16s]:	1.3
RNA Concentration:	19 ng/µl	RNA Integrity Number (RIN):	4.6 (B.02.08)

Fragment table for sample 7 : M7

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,516	1,742	1.7	3.4
23S	2,669	3,068	2.2	4.5

(11) Sample name: M8



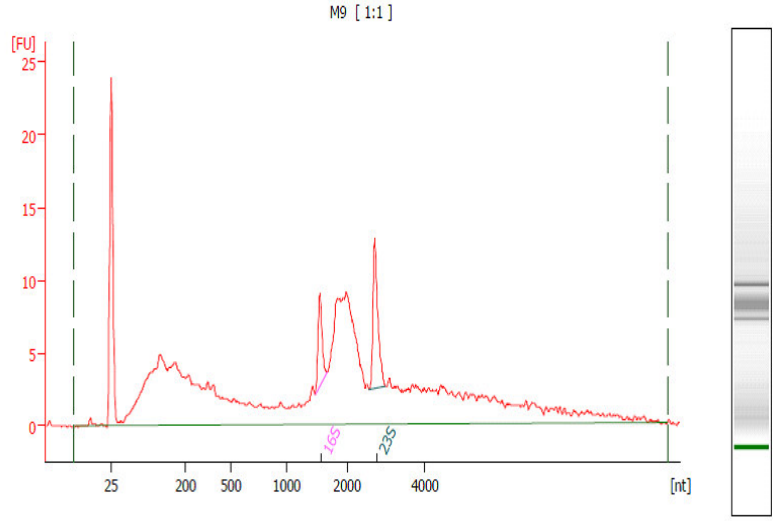
Overall Results for sample 8 : M8

RNA Area:	345.3	rRNA Ratio [23s / 16s]:	0.3
RNA Concentration:	133 ng/µl	RNA Integrity Number (RIN):	3.5 (B.02.08)

Fragment table for sample 8 : M8

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,651	1,906	16.9	4.9
23S	2,914	3,483	4.8	1.4

(12) Sample name: M9



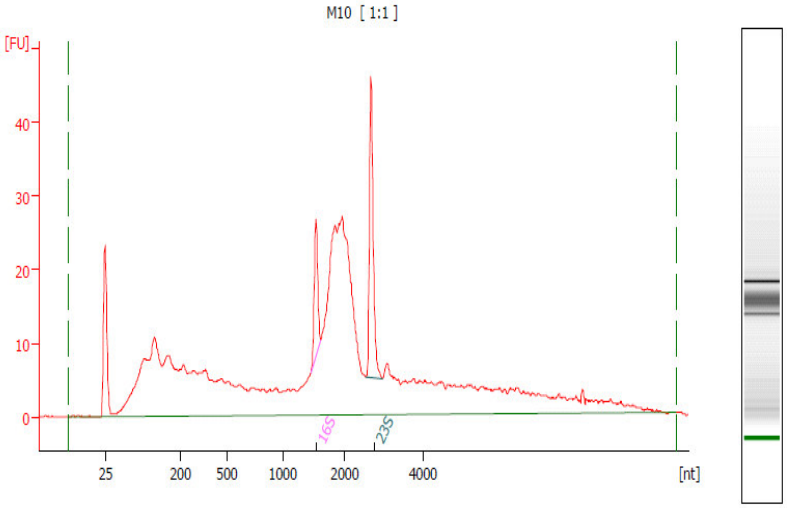
Overall Results for sample 9 : M9

RNA Area:	263.3	rRNA Ratio [23s / 16s]:	1.8
RNA Concentration:	101 ng/µl	RNA Integrity Number (RIN):	4.1 (B.02.08)

Fragment table for sample 9 : M9

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,477	1,665	6.1	2.3
23S	2,574	2,992	11.0	4.2

(13) Sample name: M10



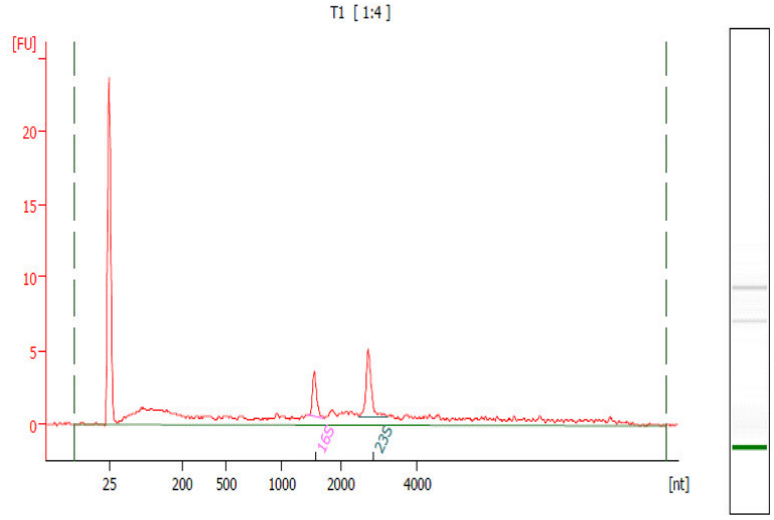
Overall Results for sample 10 : M10

RNA Area:	614.8	rRNA Ratio [23s / 16s]:	2.1
RNA Concentration:	236 ng/µl	RNA Integrity Number (RIN):	4.3 (B.02.08)

Fragment table for sample 10 : M10

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,450	1,619	16.0	2.6
23S	2,525	2,961	34.0	5.5

(14) Sample name: T1



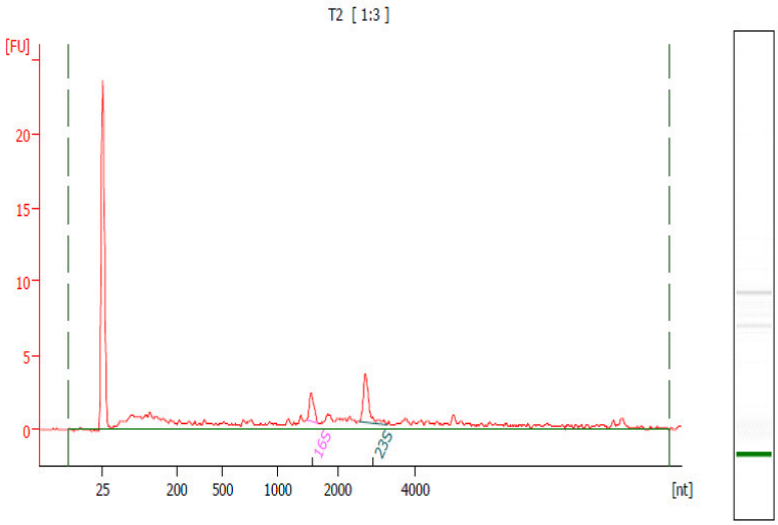
Overall Results for sample 11 : T1

RNA Area:	67.0	rRNA Ratio [23s / 16s]:	1.8
RNA Concentration:	26 ng/µl	RNA Integrity Number (RIN):	6.6 (B.02.08)

Fragment table for sample 11 : T1

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,455	1,715	3.1	4.7
23S	2,476	3,211	5.5	8.3

(15) Sample name: T2



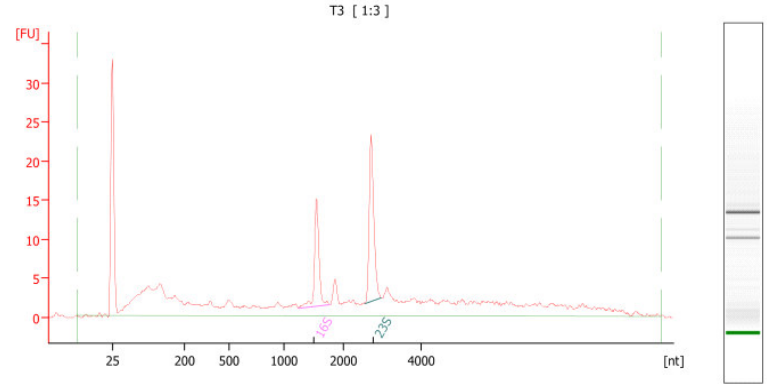
Overall Results for sample 12 : T2

RNA Area:	54.8	rRNA Ratio [23s / 16s]:	1.9
RNA Concentration:	21 ng/µl	RNA Integrity Number (RIN):	5.9 (B.02.08)

Fragment table for sample 12 : T2

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,482	1,673	2.2	4.1
23S	2,554	3,259	4.2	7.7

(16) Sample name: T3



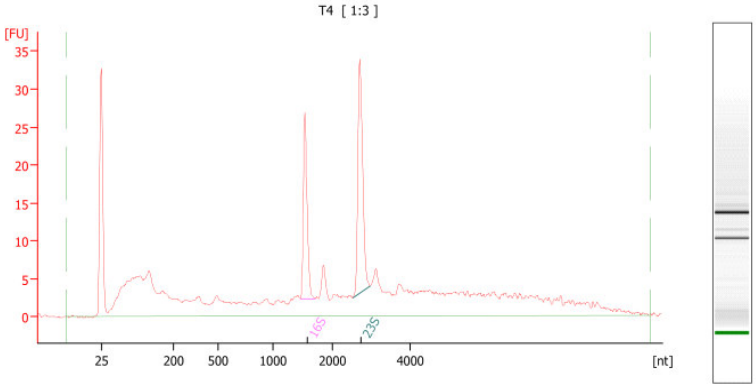
Overall Results for sample 1 : T3

RNA Area:	220.5	RNA Integrity Number (RIN):	7.2 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	55 ng/µl		
rRNA Ratio [23s / 16s]:	1.4		

Fragment table for sample 1 : T3

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,250	1,774	16.0	7.3
23S	2,550	2,961	22.3	10.1

(17) Sample name: T4



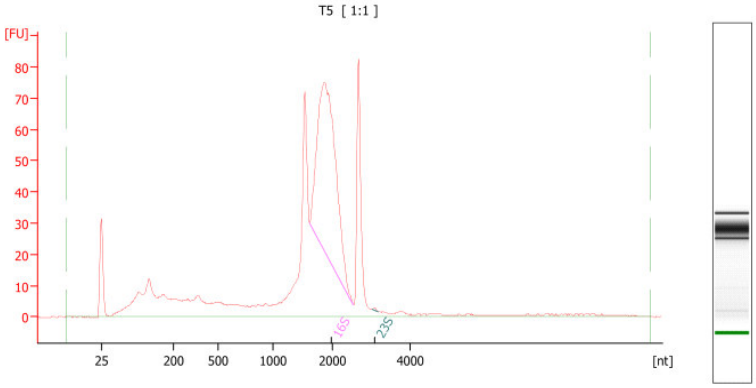
Overall Results for sample 2 : T4

RNA Area:	334.5	RNA Integrity Number (RIN):	7.2 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	83 ng/µl		
rRNA Ratio [23s / 16s]:	1.5		

Fragment table for sample 2 : T4

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,457	1,704	23.2	6.9
23S	2,519	2,976	33.7	10.1

(18) Sample name: T5



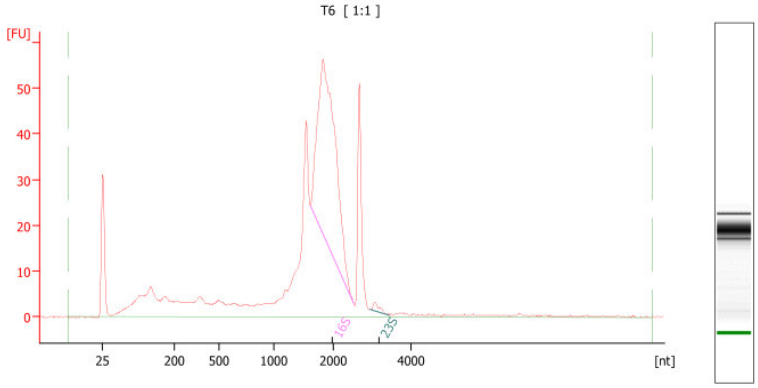
Overall Results for sample 3 : T5

RNA Area:	846.6	RNA Integrity Number (RIN):	7.4 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	211 ng/µl		
rRNA Ratio [23s / 16s]:	0.0		

Fragment table for sample 3 : T5

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,625	2,535	241.1	28.5
23S	3,022	3,174	0.5	0.1

(19) Sample name: T6



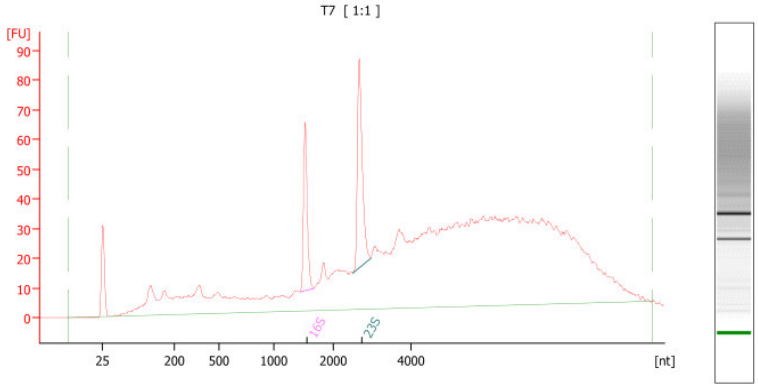
Overall Results for sample 4 : T6

RNA Area:	602.9	RNA Integrity Number (RIN):	7.4 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	150 ng/µl		
rRNA Ratio [23s / 16s]:	0.0		

Fragment table for sample 4 : T6

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,613	2,519	169.1	28.0
23S	2,946	3,450	2.8	0.5

(20) Sample name: T7



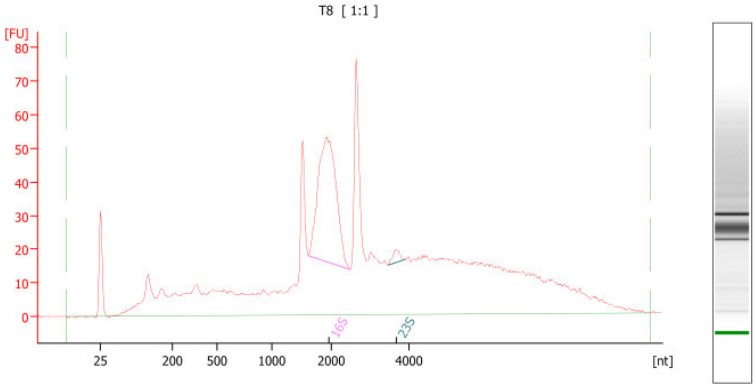
Overall Results for sample 5 : T7

RNA Area:	1,472.2	RNA Integrity Number (RIN):	6.4 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	367 ng/µl		
rRNA Ratio [23s / 16s]:	1.4		

Fragment table for sample 5 : T7

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,435	1,673	54.1	3.7
23S	2,503	2,961	77.5	5.3

(21) Sample name: T8



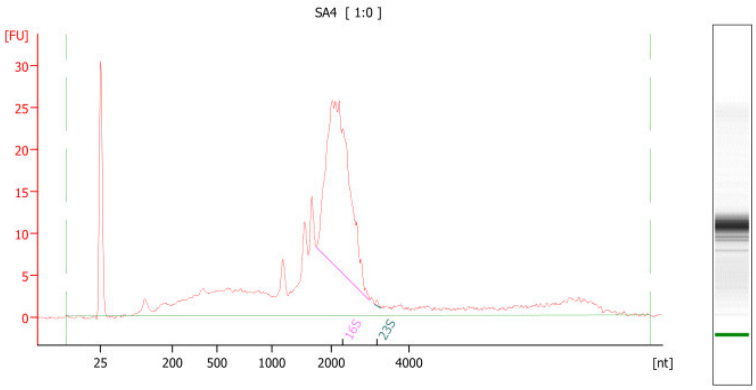
Overall Results for sample 6 : T8

RNA Area:	1,281.3	RNA Integrity Number (RIN):	5.7 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	319 ng/µl		
rRNA Ratio [23s / 16s]:	0.0		

Fragment table for sample 6 : T8

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,613	2,488	159.2	12.4
23S	3,465	3,908	6.0	0.5

(22) Sample name: SA4



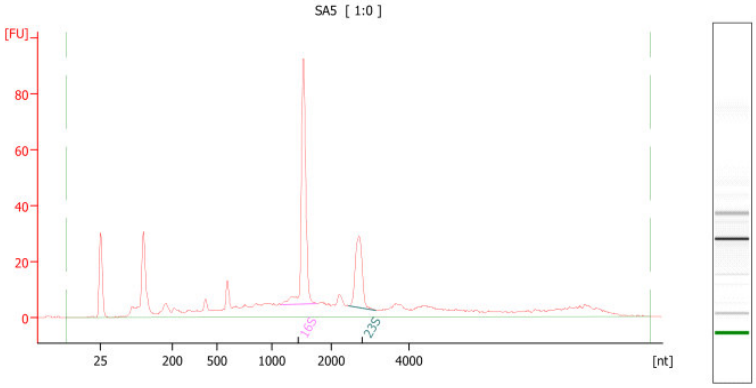
Overall Results for sample 7 : SA4

RNA Area:	338.8	RNA Integrity Number (RIN):	7.4 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	84 ng/µl		
rRNA Ratio [23s / 16s]:	0.0		

Fragment table for sample 7 : SA4

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,742	2,977	108.2	31.9
23S	3,129	3,282	0.3	0.1

(23) Sample name: SA5



Overall Results for sample 8 : SA5

RNA Area:	488.7	RNA Integrity Number (RIN):	7.7 (B.02.08, Anomaly Threshold(s) manually adapted)
RNA Concentration:	122 ng/µl		
rRNA Ratio [23s / 16s]:	0.4		

Fragment table for sample 8 : SA5

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,148	1,752	102.4	21.0
23S	2,458	3,145	41.4	8.5

Sample Name	Test Instrument	Test Kit	Dilution Ratio(×)	Test Concentration (ng/µL)	Concentration of original sample(ng/µL)	RIN	23S/ 16S	Remark
C1	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	41	41	3.0	0.6	
C2	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	68	68	2.6	0.1	
C3	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	14	14	2.5	0.0	
M1	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	42	84	3.5	0.8	
M2	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	18	36	4.4	0.4	
M3	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	45	90	2.8	1.6	
M4	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	50	100	4.1	1.3	
M5	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	46	92	5.0	0.7	
M6	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	31	62	4.5	1.4	
M7	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	19	38	4.6	1.3	
M8	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	133	266	3.5	0.3	
M9	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	101	202	4.1	1.8	
M10	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	236	472	4.3	2.1	
T1	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	5	26	130	6.6	1.8	
T2	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	4	21	84	5.9	1.9	
T3	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	4	55	220	7.2	1.4	
T4	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	4	83	332	7.2	1.5	
T5	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	211	422	7.4	0.0	
T6	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	150	300	7.4	0.0	
T7	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	367	734	6.4	1.4	

T8	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	2	319	638	5.7	0.0	
SA4	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	84	84	7.4	0.0	
SA5	Agilent 2100 Bioanalyzer	Agilent RNA 6000 nano Reagents Port 1	1	122	122	7.7	0.4	

Appendix2: Original information of sample

Sample Type:									
total RNA									
Sample status:									
Dissolved in RNase-free water (or DEPC treated water)									
Further Information:									
Sample Name	**Species**	**No. of Tubes**	**Concentration (ng/μL)**	**Volume (μL)**	**Total Quantity (μg)**	**28S/18S**	**OD260/280**	**OD260/230**	**Remark**
C1		1			0		0	0	
C2		1			0		0	0	
C3		1			0		0	0	
M1		1			0		0	0	
M2		1			0		0	0	
M3		1			0		0	0	
M4		1			0		0	0	
M5		1			0		0	0	
M6		1			0		0	0	
M7		1			0		0	0	
M8		1			0		0	0	
M9		1			0		0	0	
M10		1			0		0	0	
T1		1			0		0	0	
T2		1			0		0	0	
T3		1			0		0	0	
T4		1			0		0	0	
T5		1			0		0	0	
T6		1			0		0	0	
T7		1			0		0	0	
T8		1			0		0	0	
SA4		1			0		0	0	
SA5		1			0		0	0	

End of Report