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"PROFESSOR JOSÉ DE SOUZA HERDY"

IVANA BOGADO MARTINEZ

THE INFLUENCE OF CONDITIONS ASSOCIATED TO THE PULMONARY TISSUE OF CYSTIC FIBROSIS PATIENTS IN THE VIRULENCE OF *Burkholderia cenocepacia*

DUQUE DE CAXIAS
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Thesis presented at Universidade do Grande Rio "Prof. José de Souza Herdy", in partial fulfilment of the requirements to obtain the Doctor of Philosophy (Ph.D.) degree in Biomedical Sciences.

Concentration Area: From Biological Systems to Translational Biomedicine

Supervisor: Prof. Doctor Eidy de Oliveira Santos

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“Do not wait: the time will never be 'just right'. Start where you stand, and work whatever tools you may have at your command and better tools will be found as you go along.”

— Napoleon Hill

"J'ai appris que le courage n'est pas l'absence de peur, mais la capacité de la vaincre."

—Nelson Mandela

"This whole time, I've been living for my treatments, instead of doing my treatments so that I can live. I want to live. It's just life, Will. It'll be over before we know it"

— Character Stella, a teenager who has cystic fibrosis - Movie "Five Feet Apart"

RESUMO

Burkholderia cenocepacia é um dos patógenos mais graves associados a infecções pulmonares em fibrose cística (FC) e poucos dados estão disponíveis sobre a influência das condições pulmonares de FC em seu comportamento. Através de ensaios *in vivo* e *in vitro* avaliamos, respectivamente, a virulência e a produção de fatores de virulência de *B. cenocepacia*. Nós hipotetizamos que *B. cenocepacia* encontra-se sob estresse em pulmões de pacientes com FC, o que desencadeia um aumento em sua virulência. Como altos níveis de NaCl estresse osmótico são naturalmente associados a pulmões de pacientes com FC ou são observados durante intervenções terapêuticas (salina hipertônica), nós testamos o efeito de 0,4 M NaCl na cepa epidêmica J2315. N-acetilcisteína (NAC 1g/L), solução usada no controle de exacerbações pulmonares de pacientes com FC, também foi investigado. Em diferentes fases de crescimento, nós avaliamos a *B. cenocepacia* através de ensaios *in vitro*, produção de biofilme, adesão à mucina, inibição contra *Staphylococcus aureus* e *Pseudomonas aeruginosa* e análises de exoproteomas. Na segunda parte deste trabalho, testamos em zebrafish a K56-2, que representa outra cepa de *B. cenocepacia* com níveis semelhantes de virulência. Antes da injeção dos embriões, a *B. cenocepacia* foi cultivada em meio LB complementado com NaCl (i) ou em meio artificial que mimetiza o escarro de FC (ASMDM) (ii), ou pré-ativada através de 1h de tratamento com cisteamina injetada (iii), ou adicionada à água do banho do zebrafish (iv). Quando testados individualmente, NaCl e NAC reduziram em *B. cenocepacia* a produção de fatores de virulência, mas quando combinados, aumentaram o biofilme e a adesão à mucina. Por meio de ensaios de exoproteômica, identificamos uma proteína relacionada ao sistema de secreção tipo III, BipC, que foi obtida exclusivamente em meio LB. Nenhuma das condições testadas no *zebrafish* alterou a virulência da bactéria. Concluindo, NAC associado a NaCl potencializa a produção de fatores de virulência em *B. cenocepacia*, mas, como observado para o NaCl, isto não resulta necessariamente em um efeito no hospedeiro. A cisteamina tem sido investigada em FC por, por exemplo, inibir *B. cenocepacia* e outros patógenos. Diferente do esperado, pela primeira vez demonstramos que a cisteamina não afeta expressão de citocinas inflamatórias nem a virulência da bactéria. Esses resultados esclarecem aspectos importantes da virulência de *B. cenocepacia* nos pulmões de pacientes com FC.

Palavras-chave: *B. cenocepacia*, biofilme, adesão à mucina, competição, *zebrafish*, virulência

ABSTRACT

Burkholderia cenocepacia is one of the most serious pathogens associated with pulmonary cystic fibrosis (CF) infections, and few data are available about the influence of CF pulmonary conditions on its behavior. In this work we assessed its virulence and virulence factor production by *in vivo* and *in vitro* assays, respectively. In the first section of this work, we hypothesized *B. cenocepacia* is under stress in the CF lungs, which triggers an increase in its virulence. We tested the osmotic stress (0.4M NaCl) on the epidemic strain J2315, as major NaCl levels are naturally expected to occur in CF lungs or are associated with therapeutical intervention (hypertonic saline). N-acetyl cysteine (NAC 1g/L), a solution that has been used to control pulmonary exacerbations in CF, was investigated as well. Through *in vitro* assays, at different growth phases, we evaluated *B. cenocepacia* through biofilm production, mucin adhesion, inhibition assays against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and exoproteome analysis. In the second part of this work we tested k56-2 in zebrafish, which is another *B. cenocepacia* strain with similar levels of virulence. Before embryotic injections, *B. cenocepacia* was cultivated in LB media complemented with NaCl (0.4M) (i), or in an artificial medium that mimics the CF sputum (ASMDM) (ii) or pre-activated with 1 hour treatment with cysteamine injected into zebrafish (iii), or added to the zebrafish bathing water (iv). When tested individually, NaCl and NAC have been shown to reduce *B. cenocepacia* virulence factor produced, but when combined there was an observed increased in biofilms and mucin adhesion. Through exoproteomic assays, we identified a protein related to the type III secretion system, the BipC, that was obtained exclusively in LB medium. None of the conditions tested in *zebrafish* altered the bacterial virulence. In conclusion, NAC associated with NaCl potentiates the production of virulence factors in *B. cenocepacia* but does not necessarily result in an effect on the host. In addition, NAC associated with NaCl potentiates virulence factors production in *B. cenocepacia*, but, as observed for NaCl, this does not necessarily result in an effect on the host. Cysteamine has been investigated in CF as, for example, it inhibits *B. cenocepacia* and other pathogens. Contrary to the expected results, for the first time we demonstrated that cysteamine does not affect the expression of inflammatory cytokines or virulence of the bacterium. These results clarify important aspects of *B. cenocepacia* virulence in the lungs of CF patients.

Keywords: *B. cenocepacia*, biofilm, mucin adhesion, competition, zebrafish, virulence

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LIST OF ABBREVIATIONS AND ACRONYMS

16S rRNA 16S: ribossomal RNA 16S
ABC: ATP binding cassete
ACN: acetonitrile
ADH: yeast alcohol dehydrogenase
AHLs: acyl homoserine lactones
Apr: alkaline protease
ASL: airway surface liquid
ASM: artificial sputum media
ASMDM: modified artificial-sputum medium
ATCC: American Type Culture Collection
B cell: lymphocyte B
Bcc: *Burkholderia cepacia* complex
BCESM: *Burkholderia cenocepacia* epidemic strain marker
BDSF: cis-2-dodecenoic acid
BGN: gram negative bacilli
BSA: bovine serum albumin
CAZ: Ceftazidime
cbl: cable pilus gene
Cci: B. cenocepacia genomic island
CDS: coding sequence
CF: cystic fibrosis
CFTR: cystic fibrosis transmembrane conductance regulator
CFU: colony forming unit
CGD: Chronic Granulomatous disease
CK: cytokeratin
Cm: chloramphenicol
DAU: Double sugar urea
DNA: Deoxyribonucleic acid
Dpf: days post fertilization
DIC: differential interference contrast
DNA: deoxyribonucleic acid
DTPA: diethylene triamine Penta acetic acid
EDTA: ethylenedeamine tetra acetic acid
ENaC: epithelial sodium channel
EPS: Exopolysaccharide
ESI: electrospray ionization
esmR: epidemic strain marker regulator
ET-12: Edinburgh-Toronto epidemic clone (ET-12)
fli: flagellin gene
G3BP: Ras-GAP SH3 domain binding protein
GN: Gram-negative
GSH: glutathione
hBD: Human beta defensin
HIV: Human Immunodeficiency Virus
Hpf: hours post fertilization
Hpi: hours post infection
HS: hypertonic saline
IFN: interferon
Ig: immunoglobulin
IL: interleukin
IS: insertion sequence
Iv: intravenous
K56-2: *Burkholderia cenocepacia* K56:2
kDa: kilodalton
LB: Luria-Bertani Medium
LC3: light chain 3
LPC: periciliar liquid layer

LPS: lipopolysaccharide
M: Molarity
M9: M9 minimal medium
MALDI-TOF: Matrix Assisted Laser Desorption Ionization Time of Flight
MCC: mucociliary clearance
MEM: meropenem
MF: McFarland
MH: minocycline
MHA: Mueller Hinton Agar
MHCII: major histocompatibility complex II
MIC: minimal inhibitory concentration
MLST: multilocus sequence typing
MOT: motility
MRSA : multi-resistant *S. aureus*
MS: mass spectrometry
MS/MS: Tandem mass spectrometry
MS222: tricaine methanesulfonat
MUC: mucine
MVs: membrane vesicles
NAC: N-acetylcysteine
NK: Natural killer cell
NO: nitric oxide
O/F: oxidative / fermentative
OD: Optical Density
ODc: Optical Density
OLS: Ordinary Least Square
OMVs: outer membrane vesicles
ORFs: open reading frames
PAMP: pathogen-associated molecular pattern
PB: Polimixina B
PBS: phosphate-buffered saline
PCL: periciliar liquid layer
PF: paraformaldehyde
PHB: poli-β-hydroxybutyrate
PMN: polymorphonuclear neutrophil
PR: phenol red
PRR: pattern recognition receptor
Ptw: plant tissue watersoaking
PYR: pyrrolidonyl aminopeptidase
qRT-PCR: real-time reverse transcription-PCR
QS: quorum sensing
RCF: relative centrifugal force
RFLP: Restriction Fragment Length Polymorphism
ROS: reactive oxygen species
RqpSR: Regulating Quorum sensing and Pathogenicity
SCFM: sputum CF medium
SD: standard deviation
SEM: standard error of the mean
SIM: Sulfide, Indole, Motility
SPLUNC: short palate lung and nasal epithelial clone
SXT: Trimethoprim-sulfamethoxazole
TCA: trichloroacetic acid
Tg: transgenic
TLR: Toll-like
TSB: Tryptic Soy Broth
TTC: 2,3,5-Triphenyltetrazolium Chloride
TTSS: type III secretion system
UERJ: Universidade Estadual do Rio de Janeiro
USP: ubiquitin-specific protease
VirB: Virulence regulon transcriptional activator VirB

SUMMARY

INTRODUCTION

Cystic Fibrosis (CF) is a gravely serious disease that shortens life expectancy of people worldwide, estimated by about 40 years. Patients dedicate their whole life to dealing with symptoms associated to it, as a definitive treatment still does not exist. Although classified as a genetic disease, these patients often die of pulmonary complications. One of the feared pathogens is *Burkholderia cenocepacia*, which worsens the already bad prognosis and life expectancy of these patients. The Edinburgh-Toronto epidemic clone (ET-12) is one of the strains associated with this most severe condition (Sun *et al.*, 1995).

This pathogen is far from being the most frequent among the infecting agents, but it is the one responsible for worst outcomes related to CF infections and can lead to a rapid death. In the 1980's, for example, the ET12 clone caused high morbidity and mortality amongst patients as a result of its high transmissibility and acute necrotic pulmonary infections, known as cepacia syndrome. Lungs are the major organ compromised during cepacia syndrome (Goodlet *et al.*, 2019). This thesis work analysed parameters specifically associated with CF pulmonary tissue.

There are few studies exploring specific pulmonary conditions of CF lungs correlated to *B. cenocepacia* ET12 infection. Among variables employed *in vitro*, in this work we analysed the effect of growth phase, known to interfere with virulence (Chaffin *et al.*, 2012) and NAC, suggested as a potential strategy for CF treatment (Dauletbaev *et al.*, 2009; Conrad *et al.*, 2015), although its role is still controversial demanding further investigations.

To understand how such bacteria react to an increase in NaCl concentration during nebulization with hypertonic saline, we studied the effect of NaCl in culture media by using concentrations that exceeded the physiological concentration (0.2M and 0.4M in comparison to 1.5M or 0.9%, respectively). Previously, other groups described that presence of higher concentrations of NaCl can predispose to infections and impair immune system function (Goldman *et al.*, 1997).

In contrast, it has also been found to reduce the number of exacerbations associated with acute infections, avoiding acute and severe illness (Williams *et al.*, 2010). However, thus far, to our knowledge these studies have not been performed with *B. cenocepacia*. We also assessed

the behavior of ET12 clones by means of proteomics and biofilm formation to evaluate virulence factor production. We also included competitive *in vitro* assays to closely mimic the conditions of CF lungs. CF patients are often plagued by polymicrobial infections, and interactions between *B. cenocepacia* and other bacteria as *S. aureus* (Thompson, 2017) and *P. aeruginosa* (Bragonzi *et al*., 2012) can occur.

Finally, we have also analysed the effect of high osmolarity and NAC during mixed infections. To test the effect of NaCL during infection *in vivo*, we exploited the advantages of the zebrafish embryo infection model, developed to study virulence of *B. cenocepacia*. In addition, we have analysed the effect of ASMDM media, which was developed to mimic physical-chemical properties of CF sputum (Fung *et al.*, 2010), and cysteamine, lately found to have *in vitro* properties that may be beneficial for CF patients (Devereux *et al.*, 2015), during zebrafish infections.

LITERATURE REVIEW

1. *Burkholderia cepacia* complex (Bcc)

1.1. Bcc in the context of CF

The *Burkholderia cepacia* complex (Bcc) is a versatile group of highly related bacteria that have a ubiquitous distribution in nature (Vandamme and Peeters, 2014). This complex is a subgroup of the genus *Burkholderia*, belonging to the class *Betaproteobacteria*. In 1992, Yabuuchi *et al*. described that some species previously classified as *Pseudomonas sp*. of group II presented different characteristics related to 16S rRNA sequences, DNA-DNA homology assays, fatty acid and lipid content, and phenotypic tests.

Thus, they proposed the reclassification of these seven species for this new genus, named in honor of W.H. Burkholder, an American bacteriologist. *Burkholderia cepacia*, previously known as *P. cepacia*, was defined as the type species of this genus (Yabuuchi *et al.*, 1992; Euzéby, 1997).

A total of 122 species are sorted in this genus (Parte, 2018). It is composed of plant and animal pathogens, and environmental microorganisms as well. Recently this classification was revised, and all species belonging to *Burkholderia* genera were split and repositioned (Dobritsa and Samadpour, 2016). In this context, in the Bcc complex, 21 highly related species were grouped (Estrada-De-Los-Santos *et al.*, 2016; Martina *et al.*, 2018).

Bcc bacteria are gram-negative and saprophytic, with curved or straight bacilli morphology, oxidase and catalase positive and non-fermenting phenotypes. They are mobile bacteria, and in general, they have several polar flagella. Most species accumulate carbon granules containing reserves of poly-β-hydroxybutyrate (PHB).

In relation to the lipid content, they may present fatty acids containing 14, 16 or 18 carbon atoms. They are chemotrophic and often use oxygen as the final acceptor of electrons of the

respiratory chain. Although, some species can perform anaerobic respiration through nitrate. The Guanine + Cytosine content of the DNA ranges from 59 to 69.6 mol%. Species of this complex exhibit a high degree of sequence similarity in the gene for the ribosomal ribonucleic acid 16 subunit (16S rRNA) (98-100%) (Vanlaere *et al.*, 2009). Pigmentation is not a universal feature of this genre. They present growth at 30º C, but can also be grown at other temperatures as 37º C and, in some cases, at 42º C (Yabuuchi *et al.*, 2005).

A study carried out in 1997 distributed the strains identified as *Bcc* in five genomovars (I to V), that presents sufficient genetic differences and are detectable by simple differential assays, despite the phenotypic similarities (Vandamme *et al.*, 1997). In this study, *B. cenocepacia* was recognized as belonging to genomovar III (De Smet *et al.*, 2015; Estrada-De-Los-Santos *et al.*, 2016).

In the light of the polyphasic taxonomy, recent discussions resurged proposing new sorting in the *Burkholderia* group. After more than 80 species were grouped in the *Burkholderia* genus, multilocus sequencing typing (MLST) and other approaches indicated that actually, this was a polyphyletic genus.

The genus has been then split in *Paraburkholderia* and *Burkholderia*, and *Caballeronia*. The former, retains environmental bacteria; in *Burkholderia*, only animal and plant pathogens species were kept, and *Caballeronia*, became a distinctive clade in the phylogenetic tree, retaining species previously classified as *Paraburkholderia* (Sawana *et al.*, 2014; Dobritsa and Samadpour, 2016; Eberl and Vandamme, 2016).

Bacteria of *Burkholderia* genus can be found in soil, rhizosphere and on the interior or surface of plants and fruits (Coenye and Vandamme, 2003). They were originally described as causing infection on the onion bulb, causing it to rot (Palleroni and Holmes, 1981). Over time, its potential for biotechnology has been revealed, and today its abilities as biocontrol agents, bioremediation and plant growth promoters are well described (De Smet *et al.*, 2015). However, their pathogenic character in humans and plants prevents large scale applications.

Because they survive in aquatic environments and have high transmissibility, Bcc have gained a lot of attention when they emerged as important nosocomial pathogens causing outbreaks in hospitals and health care units. In addition to their occurrence in medical-hospital contexts, they show intrinsic resistance and persistence in the presence of many antimicrobials and antiseptics used in the treatment and control of hospital infection (Sajjan *et al.*, 2008). This phenotype and its intracellular ability to survive increase the researches concern with *Burkholderia* species related to human health.

The role of Bcc as human opportunistic pathogens is very well described, especially in infections of individuals with cystic fibrosis and chronic granulomatous disease (CGD), revealing their association in cases where immunological functions are compromised. Besides being isolated from pulmonary samples, they are also found in bacteremia and in other anatomical sites. They also can cause urinary tract infections, septic arthritis and peritonitis, for example (Jorgensen and Pfaller, 2015).

Most of Bcc bacteria can be found in lungs of CF patients. However, *B. multivorans* and *B. cenocepacia* are the most prevalent and particularly virulent (Mahenthiralingam *et al.*, 2005). Despite this, Bcc does not represent the major group associated with CF. Pulmonary infections in CF are more commonly attributed to *P. aeruginosa*, but Bcc is reported as responsible for 3.5% of CF cases in the world, and up to 9% in Brazil (Rebrafc, 2015).

Eventually, a high prevalence of *B. cenocepacia* can occur in a CF center. For instance, in a work published by Drevinek *et al*. (2005), Bcc was reported to be responsible for up to 30% of infections in a Czech Centre (Drevinek *et al.*, 2005); in a report from another reference centre in Brazil, it reached up 22.5% (Dentini *et al.*, 2017). Although Bcc infections are less common, these pathogens have been shown to cause highly severe infections, with a high rate of transmissibility and mortality, as well as a low life expectancy for CF patients (Lipuma *et al.*, 1990; Govan *et al.*, 1993; Jones *et al.*, 2004; Mahenthiralingam *et al.*, 2005; Rebrafc, 2015).

Pulmonary infections caused by bacteria of the *Bcc* show variable outcomes. Colonized patients may present as asymptomatic carriers or may develop chronic infections. Transmission of Bcc occurs through aerosol droplets through direct physical contact with infected individuals or with contaminated surfaces. Once present in a patient, Bcc is not easily eliminated, establishing a persistent infection in the host. Bcc infection is usually acquired late, and the outcome of infection can range from maintaining stable respiratory functions to a rapid clinical decline in respiratory function, often fatal.

The most severe condition is known as cepacia syndrome (Mahenthiralingam *et al.*, 2005). This syndrome, which is rarely caused by other pathogens, is characterized by necrotizing pneumonia, associated with gradual loss of lung function, and sometimes accompanied by septicemia. It is usually fatal within one year (Isles *et al.*, 1984; Lipuma *et al.*, 1990).

2. *B. cenocepacia* and the ET12 epidemic clone

Among the bacteria belonging to Bcc, *B. cenocepacia* is described as the dominant and most virulent species. This specie is recognized formally as Bcc genomovar III. This specie is highly frequent among CF patients in Brazil (Carvalho *et al.*, 2007). The most studied epidemic clone of *B. cenocepacia* is ET 12, responsible for the largest epidemic of CF patients in Canada and the United Kingdom in the 1980s and 1990s, and the second most important in the world. This strain is widely distributed, and has been isolated from patients from different countries on both sides of the Atlantic (Mahenthiralingam *et al.*, 2005).

ET-12 is distinguished from the others based on *recA* gene sequence analysis (Mahenthiralingam *et al.*, 2000), that split epidemic clones in four phylogenetical clusters groups (IIIA, -B, -C and -D). Most clinically relevant clones are grouped in IIIA, as ET-12 strain (ST28) and Czech strain (ST32), common in Canada and Europe; within group IIIB, the dominants in the United States are Midwest clone and the PHDC strain (Drevinek and Mahenthiralingam, 2010). IIIC is an environmental strain, and IIID has been found in patients in Sweden, Italy and Argentina (Manno *et al.*, 2004; Campana *et al.*, 2005).

The main characteristics of the ET-12 genome are described in Figure 1. The total size of the genome is 8,056 Mb. Previously, ET12 was originally described with three large chromosomes plus a 92-kb plasmid, but it was reviewed afterwards and chromosome 3 was actually proposed as a large virulence plasmid (Agnoli *et al.*, 2012).

In a recent paper, Higgins *et al* (2017) determined by Tn-Seq analysis the core genome of H111. Although recognized as a non- ET12 clone, it is very similar to that. In this paper, they found that only 5% of the predicted genes are essential for metabolism of *B. cenocepacia*, which corresponds to 398 genes (Higgins *et al.*, 2017). In both ET12 and H111, most of essential genes are located on chromosome 1.

ET12 genome also has insertion sequences (IS) and several genomic islands, which have been associated with the virulence of this bacterium (Baldwin *et al*., 2004). The genes present in these islands appear to be derived from mobile genetic elements such as transposons, plasmids and bacteriophages, and curiously, bacteriophage-like sequences have been identified in virtually all ET-12 strains (Summer *et al.*, 2004).

2.1. *B. cenocepacia* virulence factors (VF)

The B. cenocepacia species carry several virulence factors (some of them are represented in Figure 1). Important molecules associated with communication, adhesion, invasion, survival and activation of immune responses have already been identified in ET-12 strains. Specifically assigned to the ET12 epidemic clone, the simultaneous presence of three elements, cable pili, 22-kDa adhesin and the *B. cenocepacia* epidemic strain marker (BCESM), makes this epidemic clone unique, but other factors are shared among Bcc representatives. In common to other *B. cenocepacia* strains and even species from the Bcc group there are a lot of virulence factors and/or regulatory systems described, including quorum sensing and a type III secretion system (TTSS), flagellin recognized by Toll-like receptor 5 (TLR5), important for the inflammatory response, and others that are described below (Mahenthiralingam *et al.*, 2005; Loutet and Valvano, 2010; Mcclean *et al.*, 2016).

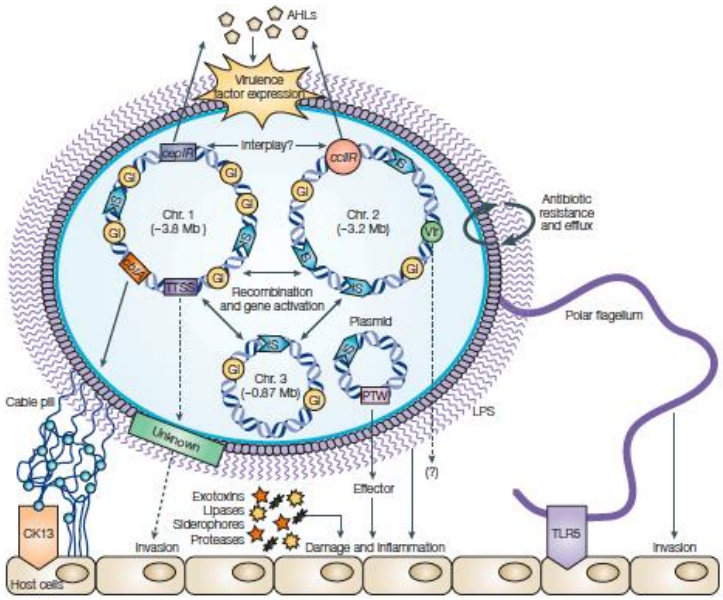


Figure 1 Genomic structure and some virulence factors of *B. cenocepacia* ET-12.
Chr.1, Chr.2, Chr3 and Plasmid, presence of genomic islands (GI) in each of the three large replicons, Multiple insertion sequences (IS)), with a brief indication of its importance in the interaction with host cells: cable pili (cblA-encoded) and 22-kDa adhesin (blue-labeled circles) the intermediate filament of cytokeratin 13 (CK13), quorum-sensing cepIR and cIRIR systems, both capable of secreting and binding to acyl-homoserine lactones (AHLs), type III secretion system (TTSS) and its possible role in invasion and *in vivo* survival, flagellin binding to the Toll-like receptor type 5 (TLR5) involved in the activation of the inflammatory response, various surface and extracellular factors, including exotoxins, lipases, lipopolysaccharides, siderophores and proteases intrinsic resistance to antibiotics present in the envelope; type IV secretion systems (PTW and VirB). Reprinted with permission from Springer Nature: Nat Rev Mmicrobiol. [The multifarious, multireplicon *Burkholderia cepacia* complex. Mahenthiralingam, E.; Urban, T. A.; Goldberg, J. B.] Copyright (2019). Source: (Mahenthiralingam *et al.*, 2005)

Lipopolysaccharide (LPS) is a classical virulence factor among gram-negative bacteria. The composition of LPS in Bcc has a specific chemical structure, and some characteristics have been correlated to bacterial resistance to cationic peptides and polymyxin, for example (Cox and Wilkinson, 1991; Shimomura *et al.*, 2003). For this reason, in the past, LPS was used for serological separation of Bcc strains. Also, its LPS is highly toxic. It has the potential to be up to five times higher than *P. aeruginosa.* It possibly explains the increase in neutrophil oxidative burst and interleukin 8 (IL-8) secretion in Bcc, but not during *P. aeruginosa* infections (Shaw *et al.*, 1995; Hughes *et al.*, 1997; Reddi *et al.*, 2003).

Biofilms are organized structures of microbial cells, capable of developing on inert materials (Mccoy *et al.*, 1981). It is now known that in infectious conditions, such as those occurring in CF, these structures are formed without the obligatory support surface, as it normally is observed (Bjarnsholt, 2013). Bacterial cells secrete exopolysaccharides (EPS) to produce a polymer matrix associated to biofilms that acts to protect against the action of antimicrobial substances and hinders the action of the host's immune system (Blasi *et al.*, 2016).

Until now, *B. cenocepacia* biofilms were not found *in vivo* in infections related to CF patients. In CF pulmonary infections caused by *P. aeruginosa* , though, biofilms have already proved to occur (Pellizzoni *et al*., 2016). *In vitro* Bc biofilms occur in static systems, in cell cultures, including epithelial cells and 3D tissues (Coenye, 2010; Van Den Driessche *et al.*, 2017). Genes related to biofilm production were also found in the genome of the bacteria (Huber *et al.*, 2002).

Instead of biofilms, *B. cenocepacia* have been recently shown to occur *in vivo* in "micro colonies or as single cells inside phagocytes or in mucus, and not in biofilm-like structures" (Schwab *et al.*, 2014). Supporting this theory, Mesureur *et al.* (2017) have shown that intracellular steps in macrophages are essential for *B. cenocepacia* infection in the zebrafish model (Mesureur *et al.*, 2017), which is in agreement with clinical observations (Schwab *et al.*, 2014) and represent a change of paradigm in this field.

Recently, in macrophages, a cluster called *afc* under regulatory control of the LysR-type transcriptional regulator ShvR was found to induce the production of a lipopeptide with antifungal activity. Its role was also associated to the transition from intracellular persistence to acute disseminated inflammatory disease (Gomes *et al.*, 2018). Findings related to this system corroborate that intracellular steps are essential for *B. cenocepacia* infection of zebrafish.

Several secretion systems have been described in *B. cenocepacia* (type II secretion system to type VI secretion system; T2SS, T3SS, T4SS, T5SS and T6SS) (Depluverez *et al.*, 2016). In *Bc*, T2SS was found to deliver two zinc-dependent metalloproteases, ZmpA and ZmpB, that cleave antimicrobial peptides involved in immunological response, as cathelicidin LL-37 and beta-defensin-1, respectively (Kooi and Sokol, 2009). Two T4SS were described in *B. cenocepacia*; the first, Ptw T4SS, secrets cytotoxic proteins that causes plant tissue watersoaking (PTw) and is also suggested to be important survival in eukaryotic cells (Sajjan *et al.*, 2008); the second is designated VirB/D4 T4SS, and shares with Ptw T4SS the DNA transfer ability (Zhang *et al.*, 2009).

T5SS is known to secrete virulence factors, to mediate cell-cell adhesion and to participate in biofilm formation, and up to now, at least four type V secretion proteins with hemagglutinin and pertactin domains have been detected on Bc J2315 genome (Holden *et al.*, 2009). T6SS was also described in Bc: it is composed of complex associated to the membrane and a kind of contractile phage tail, important for its pathogenesis and known to secret antimicrobial compounds against other bacteria during polymicrobial infections (Basler *et al.*, 2012; Ho *et al.*, 2014; Spiewak *et al.*, 2019).

Quorum sensing (QS) is a cell-cell communication system capable of coordinating a collective behavior through the secretion of acyl-homoserine lactone molecules (AHLs) (Abisado *et al.*, 2018). This is common to many Gram-negative bacteria and are released to modulate the genetic expression of neighboring cells. This phenomenon is dependent on cell density and can be sensed by accumulation and recognition of self- (and non-self) produced signals.

In Bcc, *N*-acyl homoserine lactones (AHLs) make up the major AHL system associated to bacterial virulence (Mccarthy *et al.*, 2010; Schmid *et al.*, 2012). This QS system is homologous of the LuxR and LuxI proteins of *Vibrio fischeri*. QS-cepRI system consists of the acylhomoserine lactone (AHL) synthase CepI and the transcriptional regulator CepR, which becomes activated after binding to AHL. The expression of virulence factors such as siderophores, lipases, proteases and those related to motility, biofilm stability and also interspecific communication mechanisms with other bacteria seems to be dependent on signaling triggered by this mechanism (Loutet and Valvano, 2010).

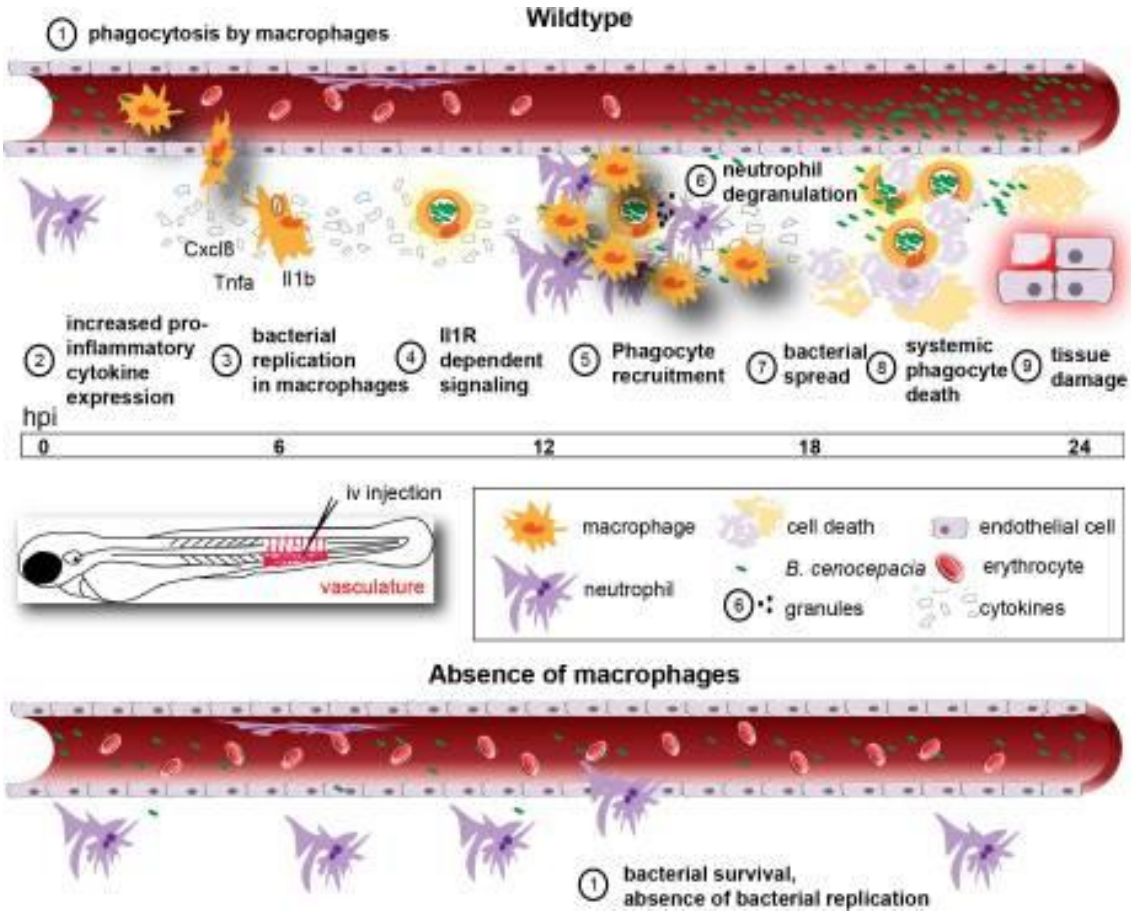


Figure 2 Schematic showing the role of macrophages during acute infection by *B. cenocepacia* K56-2 in zebrafish embryos.

Macrophages are the major phagocytosing cells of iv injected bacteria (1) for which they provide a critical replication niche (3). In their absence, K56-2 does not replicate (bottom panel, 1). Intracellular bacteria induce a rapid and robust increase in pro-inflammatory cytokine expression (2). Il1 signalling contributes to fatal pro-inflammatory responses (4), characterized by massive neutrophil and macrophage infiltration (5) neutrophil degranulation (6), bacterial dissemination from infected cells (7), systemic phagocyte death (8), and tissue damage (9). Reproduced from Mesureur J, Feliciano JR, Wagner N, Gomes MC, Zhang L, Blanco-Gonzalez M, *et al.* (2017) Macrophages, but not neutrophils, are critical for proliferation of *Burkholderia cenocepacia* and ensuing host-damaging inflammation. PLoSPathog 13(6): e1006437. Available at https://doi.org/10.1371/journal.ppat.1006437 Source: (Mesureur *et al.*, 2017)

CepRI is fully conserved within Bcc, but there are some strain-specific QS systems, as the *B. cenocepacia* genomic island (cci), for example, only associated with ET12 epidemic lineages. CciIR is a transcriptional unit composed of two predicted genes related to QS, AHL synthase gene (cciI) and a response regulator (cciR), that represents an additional QS system in Bc (Malott *et al.*, 2005).

Still, there are some Lux homologous proteins indirectly related do AHL synthase in *B. cenocepacia*, known as “unpaired” or “orphan” proteins, that help in control of QS. Usually, AHL synthase genes and their receptors are located in close proximity as revealed by whole

genome sequencing, but there are others, as cepR2 that is not in close proximity but is considered an LuxR orphan homolog, what means that it is not associated with, but requires AHL to function, as detected in *B. cenocepacia* H111 and K56-2 strains (Malott *et al.*, 2009; Ryan *et al.*, 2013).

Recently, another two-component system, RqpSR (Regulating Quorum sensing and Pathogenicity), was described to affect motility, biofilm formation and virulence in Bc. This system is known to regulate QS and pathogenesis in *B. cenocepacia* (Cui *et al.*, 2018) and mechanisms and molecules involved in communication between bacteria have stated to become clearer. RqpR controls genetic expression, as it binds to signal synthase promoters as *cis*-2-dodecenoic acid (BDSF) and AHL. This study shows the existence in *B. cenocepacia*, of a complicated hierarchy between different QS systems (Cui *et al.*, 2018).

BCESM is a virulence marker composed of a 1.4-kb fragment of DNA that is facultative in ET-12 epidemic strains. It is present in all group IIIB, and 25% of IIIA Bc strains do not have this marker (Baldwin *et al.*, 2004). In Bc K56-2, for example, it is absent, but it is also present in some environmental strains as MCO-3. It is part of a genomic island that contains genes related to virulence and pathogenicity (Mahenthiralingam *et al.*, 1997; Baldwin *et al.*, 2004). BCESM DNA fragment encodes a single putative CDS transcriptional regulator called esmR (Mahenthiralingam *et al.*, 1997).

Although it is unique to *B. cenocepacia*, it is not indicative of infection and transmissibility, but is associated with extremely complicated conditions capable of increasing mortality. It presents a varied prevalence in different countries. In Canada, it was identified in 80% of strains isolated; in the USA, only 23% (Lipuma *et al.*, 2001; Speert *et al.*, 2002). In addition to encoding virulence genes, such as those related to QS cciIR, *B. cenocepacia* has also other microbial metabolism associated with amino acid and fatty acid biosynthesis, for example.

There is a great difficulty in treating infections caused by Bcc because these microorganisms are intrinsically resistant to the antibiotics most clinically used (Nikaido and Pagès, 2012), including aminoglycoside, quinolone, and β-lactam (Chernish and Aaron, 2003). They are also capable of using penicillin G as a carbon source (Beckman and Lessie, 1979).

In Bcc, antimicrobial resistance may be caused by many reasons: reduction of membrane permeability, integrons, target changes to antibiotics and activation of efflux pumps (Tseng *et al.*, 2014). For example, in Bcc, 12 efflux pumps are recognized. The perception that the exclusive use of antibiotics is not enough to eradicate these microorganisms started a

discussion about the use of, alternative or complementary to antibiotics therapies (Blasi *et al*., 2016).

Bcc bacteria can produce five different types of pili, but only one is associated with epidemic strains, the cable pili (Cbl). The Cbl is composed of a large peritrichous protein containing 2 to 4 nm, which appears to be responsible for the arrangement of cells in clusters. It also expresses a protein of 22-kDa associated with Cbl, which seems to bind to cytokeratin 13 (CK13) present in host cell membrane, and which is recognized as the region responsible for receptor binding specificity (Sajjan *et al.*, 2003). The production of the Cbl requires the activation of 4 genes, *clbA*, *cblB*, *cblC* and *cblD*. The expression of Cbl seems to be associated with adhesin expression, and the lack of one of these compounds compromises the binding, invasion and access to the squamous epithelium (Sajjan *et al.*, 2003).

Members of the Bcc complex express at least one of the two types of flagellins, which may be differentiated based on the molecular weight of their subunits. Type I has 55 kDa, and type II (or RFLP standard of the *fliC* gene), 45 kDa. It has been show by *in vitro* studies that these structures are important for bacterial adhesion but that their presence is not critical to keep bacterial infection levels in the lung. On the other hand, its effect seems to be related to the induction of a marked inflammatory response via receptors of the Toll 5 type (TLR-5), which are determinants for the pulmonary damages observed in the CF pictures. Flagellin has also been proposed as a marker for predisposition of sepsis (Drevinek and Mahenthiralingam, 2010).

Lastly, in *B. cenocepacia*, pC3 is a large plasmid that is important for virulence in different animal infection models, including *Caenorhabditis elegans*, *Galleria mellonella* and zebrafish larvae. The plasmid-encoded genes are involved in bacterial versatility to ocupy diverse niches and resistance to various souces of stress, and it has been shown to have different properties, as fungicidal and proteolytic properties, for example (Agnoli *et al.*, 2014). The plasmid was not essential, as it could be cured from most analysed Bcc strains (Agnoli *et al.*, 2012). Only in K56-2 the plasmid was stably maintained, caused by the presence of a Toxin/antitoxin system (Agnoli *et al.*, 2014).

3. Cystic Fibrosis (CF)

3.1. General Aspects of the disease

CF is a genetic multiorgan disease that affects several organs, as the either upper and lower airway, gastrointestinal and reproductive tracts and endocrine systems (Davis, 2006). Among genetic disorders, it is responsible for the highest number of deaths in the Caucasian population (Burgel *et al.*, 2015). Its occurrence in Brazil is estimated at 1:10.000 live birth, but it can vary according to the region (Bernardino *et al.*, 2000). Clinical manifestations of CF occur due to the absence or malfunctioning of the cystic fibrosis transmembrane conductance regulator (CFTR), which compromises the transport of ions in the cell membrane of different tissue types, causing diverse physiological alterations.

The *CFTR* gene spans 250 kilobases and is located in the chromosome 7 (Zielenski *et al.*, 1991). This gene codifies 1480 amino acids in the mature protein (Zielenski *et al.*, 1991), and more than 2000 different types of mutations have been described, as reported in the Cystic Fibrosis Genetics Database (Population variation of common cystic fibrosis mutations. The Cystic Fibrosis Genetic Analysis Consortium, 1994). The most common mutation related to disease is the deletion of the residue Delta F_{508} -ΔF_{508} (Kerem *et al.*, 1990), which accounts for about 30% to 80% of all mutant alleles worldwide (Moskowitz *et al.*, 2008). Traditionally, CF mutations are classified in 6 classes (I-VII), and the F508del is classified in the group II (Veit *et al.*, 2016). Recently, the class I was proposed to be splitted in two, and one of them was named class VII (Marson *et al.*, 2016).

In CF, pulmonary outcomes are more severe, and evolve, in most cases, until death follows (Cantin, 1995; Mahenthiralingam *et al.*, 2005). Dehydration and a consequent increase in mucus thickening favor the establishment of bacterial infections. Infections are responsible for the occurrence of chronic inflammatory conditions, which can progress to respiratory failure and premature death (O'sullivan and Freedman, 2009; Wijers *et al.*, 2016).

CFTR is an ion channel of the ABC transporter family, and mutations in CFTR result in folding failure, inadequate subcellular localization and/or total protein shortage. CFTR defects observed in CF patients cause an imbalance in the homeostatic levels of sodium,

chloride and bicarbonate ions. It affects the epithelial surface of the airways leading to Airway superficial fluid (ASL) dehydration and up to 15% increase in relation to normal in mucus concentration present at this site (Zabner *et al.*, 1998; Fahy and Dickey, 2010). The mechanisms involved in this process are schematized in Figure 3.

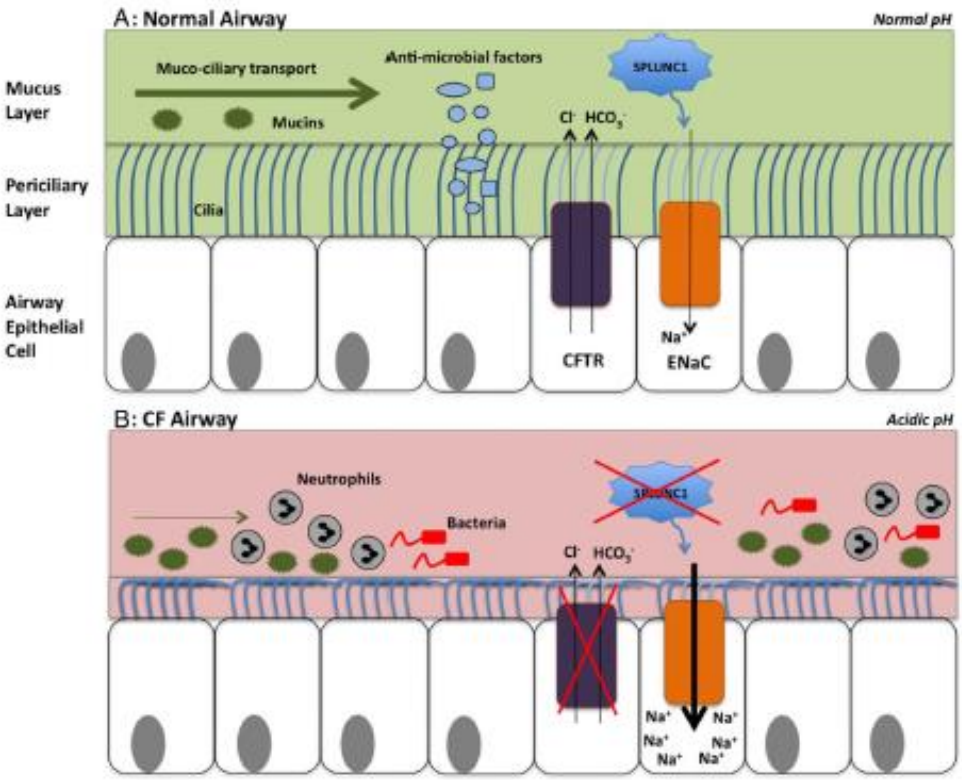


Figure 3 ASL in cystic fibrosis (CF)
(A) The normal epithelium: CFTR and MCC activity under normal hydration conditions, acting as a chloride and bicarbonate channel and contributing to the maintenance of normal pH and to the removal of inhaled pathogens on the surface of the respiratory epithelium, respectively Normal hydration of the epithelium due to the presence of mucin Immune action of antimicrobial factors secreted by cells. Normal regulatory effect of SPLUNC1 and CFTR on the sodium channel ENaC, maintaining basal intracellular sodium levels and physiological hydration of ASL. (B): Patient airways with CF: failure to transport chloride and bicarbonate by abnormal CFTR dehydrates and acidify pH. Changes in pH inhibit the activity of SPLUNC1, which becomes unable to regulate ENaC, and triggers u sodium hyperabsorption, with reduction in ASL volume; and reduce the function of antimicrobial substances, contributing to high mucus viscosity. Production of thick mucus, mucopurulent secretions, contributing to bacterial colonization. In the dehydrated state, an increase in mucus concentration occurs as the water preferably moves out of this layer. The PCL volume is initially preserved, but gradually reaches minimum thresholds, and also suffers dehydration. The mucus layer compresses PCL, compromising ciliary activity and muco-ciliary clearance (MCC). Adapted by permission from BMJ Publishing Group Limited. [Airway surface liquid homeostasis in cystic fibrosis: pathophysiology and therapeutic targets. HAQ, I. J. *et al.* v. 71, n. 3, p. 284-7 copyright 2019].Source: (Haq *et al.*, 2016)

Under normal conditions, ASL possesses large amounts of gel-forming mucins. The role of mucins is related to retention of inhaled particles by MCC. This is characterized by the proximal propulsion of particles deposited in the upper airways through ciliary movement (Knowles and Boucher, 2002). Also, the excess of mucin in this region forms a plot capable of preserving the volume of PCL.

Ions such as chloride and bicarbonate are transported through the CFTR, and sodium is internalized into the cytoplasm through another surface protein that functions as a channel and, like CFTR, also acts in the control of ASL, called sodium channel (ENaC). The regulation of sodium levels through the ENaC contributes to the muco-ciliary clearance (MCC) and to the hydration of the ASL. SPLUNC1 is another protein that is also found in ASL, which similarly to CFTR, also acts in the inhibition of ENaC. Antimicrobial substances secreted by cells present in the epithelium contribute to particle removal through innate immunity (Haq *et al.*, 2016).

In CF patients, failure to transport chloride and bicarbonate due to the presence of modified CFTR leads to dehydration and a reduction in pH. Due to pH change, SPLUNC1 loses the ability to regulate ENaC, leading to sodium hyperabsorption and depletion in ASL volume. The acid environment also reduces the function of antimicrobial substances present in the ASL, increasing the viscosity of the mucus. PCL is critical for the removal of particles such as bacteria, as it provides hydration of the pulmonary mucus and serves as a substrate for ciliary movement. Overall, CFTR depletion results in a loss of chloride secretion and an increase in sodium internalization (due to an increase in ENaC activity). The combined effects of CFTR loss and ENaC derepressing are the reduction of ASL weight, associated mucosal thickening and ciliostasis (Ballok and O'toole, 2013).

Increased viscosity, elasticity and adhesion of the mucus to the wall of the respiratory tract, make it difficult to remove and prevent cleaning of secretions, leading to an increase in lung damage (Button *et al.*, 2018). The presence of thick mucus with mucopurulent secretions favors bacterial colonization. New components derived from the necrosis of inflammatory cells (DNA and IL-8) and resulting from tissue damage (actin, elastins, cellular debris and exoproducts) also contribute to the alteration of this microenvironment.

In the dehydrated state, the water preferably moves out of the mucus layer, increasing its concentration. At the onset, the PCL volume is preserved, but during disease progression it may reach a minimum threshold at which water also leaves the PCL. The mucus layer compresses PCL, compromising ciliary activity and MCC (Fahy and Dickey, 2010; Duncan *et al.*, 2016; Wijers *et al.*, 2016).

3.2. CF mucous as an attractive site for pathogens

Mucus is a complex hydrophilic gel that retains its shape; it is deformable, elastic and viscous. In healthy individuals, the most expressive components are water (97%) and solids (3%). The solid fraction is formed mainly by mucin, but also has non-mucin proteins, salts, lipids and cell debris (Fahy and Dickey, 2010). Mucins are glycoproteins containing highly glycosylated domains, consisting of sialic acid and sulfate residues that associate with the inhaled particles. They are produced by mucoid cells that make up submucosal glands or by secretory cells located in the surface epithelium of the airways. In addition to mucus, these cells also produce other substances that can be incorporated into it, such as antimicrobials (defensins, lysozyme and surface immunoglobulins of type A-IgA) and immunomodulators (cytokines) (Fahy and Dickey, 2010).

MUC5AC and MUC5B are the most common mucins, and they are associate to a reinforced mesh through calcium-dependent covalent bonds (Henke *et al.*, 2007; Ehre *et al.*, 2012). Its glycine side chains store large amounts of liquid, up to a hundred times its weight, and allow the mucus to act as a lubricating gel and liquid reservoir of the periciliar layer. Mucus hydration is related to its viscosity and elastic properties, which in turn interfere with the effectiveness of its cleaning through ciliary action and cough (Fahy and Dickey, 2010).

Mucus functions include the capture of foreign particles and inhaled pathogens, and the ability to dissolve toxic gases, facilitating their removal from the lungs (Knowles and Boucher, 2002). In addition to act as a physical barrier to retain inhaled antigens, mucin, by means of MCC, possess another immunological role (Thornton and Sheehan, 2004; Fahy and Dickey, 2010). Together, these mechanisms provide immunological and associated protection in the elimination of inhaled particles, pathogens, and chemicals that may damage the lungs (Fahy and Dickey, 2010).

Mucus represents only one of the fractions of ASL. It is composed of a thin layer of fluid that covers the lumen (apical surface) of the upper airway epithelium. In addition to the mucus that is located more externally, ASL is also composed of an inner region called pericardial fluid (LPC) (Knowles and Boucher, 2002). This layer is adjacent to the epithelial cells that surround the respiratory cilia, and is approximately 7 um thick under normal conditions. The depth of

the mucus in turn varies between 0.5-5μm, being larger in the distal regions (Fahy and Dickey, 2010).

Under normal conditions, the mucin present in the lumen is responsible for the hydration of the LPC, and along with other factors, regulates the thickness of this layer of the ASL. In contrast to what happens in the respiratory mucus of a normal individual, the physical properties of the sputum of a CF individual are not only associated with the mucin effect but depend on a more complex mechanism of interaction between free mucins and other components including surface mucins, DNA, inflammatory cells, bacteria and changes in the ionic concentrations of other compounds. As a result, the MCC that occurs through a coordinated cilia-beating movement is compromised. However, the influence of these different types of compounds on the aberrant rheological properties of the sputum is still not well understood (Ehre *et al.*, 2014). Thus, despite its unquestionable protective effects, changes in mucus composition are related to the pathogenesis associated with CF (Haq *et al.*, 2016).

4. Conditions explored in this study associated to CF

4.1. Polymicrobial infections

Presence of microbial inhabitants in CF lungs represents another factor important for the pulmonary environment of CF patients. By traditional techniques employed for diagnostic, it was defined that during their first years, CF patients are colonized with *S. aureus* and *Haemophilus influenza*; after these early stages of life, *P. aeruginosa* is the most frequently isolated bacterial species. Infections with Bcc bacteria usually occur in adulthood, although infection with certain Bcc species have been acquired at young ages (Harrison, 2007). Presence of Bcc contaminants in CF centres have also been reported, which arise concerns about infection control (Rowbotham *et al.*, 2019).

This aparent effect related to the succession of species is partially associated to bias given by culture depended techniques currently recommended for clinical diagnostic. These techniques aim to identify a limited number of pathogens, and are directed to the ones already known to be associated to CF; they are adapted according to the clinical status of disease and

the patient age, as most frequent microorganisms associated to CF lungs vary according to these parameters (Rogers *et al.*, 2010). For example, the screening of clinical samples of CF patients in early stages of life aims in identifying *P. aeruginosa*, that is considered life-threatening; at late-stages of life, however, this practice changes as the diagnose presupposes a chronic infection, and consequently, presence of more than one specie (Rogers *et al.*, 2010).

Therewith, many microorganisms that were not recovered by these traditional cultivation methods have gone unnoticed for a long time. Recently, based on culture-independent approaches, such as high DNA throughput sequencing, the concept about CF infections has revolutionized (Héry-Arnaud *et al.*, 2019).

The presence and impact of polymicrobial communities in human disease has recently gained enormous attention, including in CF (Sibley *et al.*, 2006). In this context, climax and attack microbiomes were characterized, indicating prevalent microorganisms associated with CF patients during, respectively, clinically stable or early lung colonization and pulmonary exacerbation periods (Layeghifard *et al.*, 2019). Furthermore, factors were found to be important to determine its composition as (i) stage of infection (acute or chronic); (ii) oxygen levels (hypoxia tot anoxia); (iii) host immune response; (iv) presence of antimicrobials produced in response to competition among microorganisms; (v) antibiotics used for treatments; and (vi) the original site from where microorganism gained access to the infection (Filkins and O'toole, 2015).

The microbiomes from CF patients are not as diverse and rich as communities found in healthy individuals (Zemanick *et al.*, 2017). During disease development, for instance, microbiomes are affected by age, by antimicrobials used for treatments and they are also impacted by natural progression of chronic infection (Coburn *et al.*, 2015; Carmody *et al.*, 2018).

Some interesting behaviors between microorganisms have been observed as reviewed by Peters *et al.* (Peters *et al.*, 2012). In this work, the authors discussed detailed systems that enable crosstalk between Bcc x *P. aeruginosa* and *Candida albicans x P. aeruginosa*; infection by *S. aureus*, in contrast to *B. cenocepacia* infection, was found to increases life expectancy of CF patients around 5 years. This effect was hypothesized to prevent *P. aeruginosa* infection (Peters *et al.*, 2012). *In vitro*, *P. aeruginosa* biofilm showed to be altered when this species was co-cultivated with *B. cenocepacia*, both in regard to architecture and increased biomass (Bragonzi *et al.*, 2012).

In case of presence of at least one biofilm- producing microorganism, the picture can be worsened. Considering the diverse and complex composition and microbial community associated with biofilms, the infection becomes more difficult to be eliminated. For example, it is widely accepted that *P. aeruginosa* and *S. aureus* are microorganisms capable of forming biofilms (Maurice *et al.*, 2018; Suresh *et al.*, 2019), and a possible co-infection with one of these microorganisms *in vivo* complicates treatment of those patients.

For this reason, it is important to address questions related to CF considering also a possible interaction among microorganisms in the context of polymicrobial or co-infections, as this interaction can be key for pathogenicity. It should be noted that it is important to study infection mechanisms of individual bacterial species and in co-infections in more detail. Current evidence suggests that the intracellular life stage of opportunistic Bcc bacteria (Mesureur *et al.*, 2017) may have important roles in chronic infection and also in acute pro-inflammatory stages of infection.

More clinical studies that aims at identify infection mechanism of CF pathogens are needed to elucidate the role of co-infection in CF.

4.2. ASMDM to mimick CF sputum

Since the first attempts to cultivate bacteria *in vitro*, culture media represent one of the bottlenecks in the microbiology field. This is because we fail to identify all essential conditions needed to grow bacteria in the laboratory. In general, it is very difficult to reproduce conditions found in the original environment of microorganisms, and this is the reason it is estimated that only less than 1% of total diversity of Earth is represented by cultivable bacteria (Amann *et al.*, 1995).

Despite strong limitations of traditional methodologies to keep a wide range of microorganism in laboratory conditions, media have been developed to mimic conditions found in humans. Blood agar is an example of this and represents one of the most used culture media for diagnostic purposes. As the name suggests, blood is one of the compounds employed to prepare this medium which can be obtained from different hosts (Russell *et al.*, 2006). This medium contains factors essential for growth of microorganisms associated with blood

infections (sepsis) and enables to obtain information about hemolytic ability of microorganisms.

In case of complex environments inhabited by microorganisms, that undergoes strong modifications in response to the development of disease, as in the case of lung mucus of CF patients, efforts to reproduce these conditions *in vitro* are an important step in better understanding the development of the disease. For this reason, it is very important to develop media similar to conditions found in the host to study virulence mechanism.

In this context, to mimic sputum of CF patients ASMDM was developed that resembles the composition and physical properties of CF mucus (Fung *et al.*, 2010). It includes DNA (sperm salmon), porcine stomach mucin, potassium chloride and sodium chloride, diethylene triamine pentaacetic acid, casamino acids, bovine serum albumin, Egg yolk emulsion and antibiotic, and the pH is adjusted to 6.5 (Fung *et al.*, 2010). This final composition was adapted from ASM+, originally proposed by Sriramulu *et al.*, and adapted based on recent findings related to CF (Sriramulu *et al.*, 2005).

Among these modifications, they excluded the need of CF sputum, increased concentration of porcine stomach mucin, included albumin and lowered herring salmon sperm DNA for CF sputum levels (Sriramulu *et al.*, 2005). These conditions were then gathered in a unique media, that is now available to be used in studies addressed to better understand the effect of CF lung`s composition on virulence, and on intracellular aspect related to the behavior of the bacteria.

4.3. Salt stress in CF lungs

4.3.1. The old controversy about sodium chloride (NaCl) in CF

The airways of CF patients accumulate an increased content of mucus, suffer remodeling and are marked by a higher inflammatory response, than airway from healthy individuals. The precise mechanisms that explain the higher susceptibility to infections found in CF patients are still not completely solved (Massip-Copiz and Santa-Coloma, 2018).

One of the hypotheses to explain higher susceptibility to infections is related to levels of Na+. Some of the mechanisms under these conditions are known. Airway epithelia can absorb Na+ via ENaC, an epithelial sodium channel associated to a hyper absorption of this ion and secrete Cl^- via CFTR. It has been described that non-functional CFTR impairs the functioning of ENaC, responsible to keep physiological levels of Na+ (Haq *et al.*, 2016).

When this channel is not working properly, there is an accumulation of bicarbonate, which compromises the mucociliary clearance, associated to the removal of pathogens and other strange particles. The function of ENaC can also be regulated by a transporter associated to the airway surface, called SPLUNC, which is sensitive to pH. As the CFTR compromises transport of HCO3- and Cl, pH drops activating SPLUNC and intensifying influx of Na+. Dysregulation of these systems is associated to low water content and consequently high viscosity (Haq *et al.*, 2016).

For almost two decades, concentration of sodium chloride during pulmonary CF was a controversial matter (Zabner *et al.*, 1998; Tarran *et al.*, 2001). Based on this impasse, two hypotheses were raised to explain possible changes in the salt levels of NaCl. In the hypothesis of "high salt concentrations", it was believed that there is an increase in salt concentrations.

It was reinforced by the findings that in CFTR mutant individuals, loss of this channel transport efficacy was describe to maximize sodium absorption, and increase NaCl levels (Zabner *et al.*, 1998). This leads to the inhibition of a naturally secreted antimicrobial protein known as human beta defensin (hBD-1) that would be sensitive to salt, which would leave the epithelium FC more susceptible to infection (Smith *et al.*, 1996).

Conversely, an alternative hypothesis suggests that the ASL of CF would be isotonic, but that it would present a reduced volume. The effect of CFTR would be related to the control of essential steps of chloride secretion and sodium absorption by ENaC, a sodium absorption channel. The "low volume" hypothesis suggests that in the absence of CFTR, the osmotic force from the transport of water to the lumen is decreased and the total mass of NaCl is reduced. As consequence, there would be ASL dehydration and MCC would be delayed (Clunes and Boucher, 2007).

In support of this hypothesis, the evidence suggests that Na + and Cl- concentrations would not be different between CF and normal ASL, but rather isotonic, and that the relationship between ASL volume / thickness in the CF epithelium, *in vitro*, is lower (Jayaraman *et al.*, 2001; Song *et al.*, 2003). Also, in an *in vitro* non-invasive study with cultures of bovine tracheal epithelial cells, ASL was found to be an isotonic environment (Jayaraman *et al.*, 2001).

These data were confirmed later by experiments using trachea of mice and fresh human bronchi (Jayaraman *et al.*, 2001). In the last decades, there was an accumulation of results based on models using clinical studies, bronchial cells and mice that favored the low volume hypothesis (Clunes and Boucher, 2007).

These data agree with the conclusion of work developed by Marquette & Luckle (2016) who reviewed 09 studies based on NaCl levels in human CF lungs during infection. infected in humans in which results regarding NaCl levels in CF lungs were discrepant (Marquette and Luckie, 2016). Authors concluded these differences in NaCl levels between CF patients and their normal counterparts detected in the past (in favor of the high salt hypothesis) were in fact caused by bias related to the methodology used. For example, to measure NaCl in ASL, in all 03 studies cited as reference, the authors employed the filter paper technique to collect ASL fluid. In this case, results obtained could be compromised by fluid wicking from outside delimited areas on the paper. Then, real amount of NaCl on ASL cannot precisely be determined.

As this was the only technique used in the 3 studies analysed , they supposed this difference did not correspond to what happens in organisms (Marquette and Luckie, 2016). The same was found for ASL depth, as they did not find an effect on supporting that theory. Differences previously observed between CF an non-CF individuals might be correlated to different methodologies used (Marquette and Luckie, 2016).

Recently, to explain why CF lungs are preferred for bacterial colonization, a new hypothesis proposes that not directly NaCl nor ASL depth, but the pH (known to be acidic in CF) was the cause of abnormalities associated to bacterial infection and inflammation (Marquette and Luckie, 2016). This is supported by the original finding that CFTR not only transports Cl^-, but also bicarbonate, and that this compound affect pH on ASL (Welsh and Smith, 2001; Joo *et al.*, 2006). However, in relation to pH, differences in the results obtained in these studies are not conclusive (Song *et al.*, 2003; Song *et al.*, 2009) .

Currently, this topic still is not completely solved (Massip-Copiz and Santa-Coloma, 2018). In a recent clinical trial based on a cohort with a total of 57 patients and 62 controls, NaCl levels and pH in respiratory secretions were measured to look for secondary modifications in relation to CFTR functioning. In contrast to the last proposed theory, researchers did not detect any difference regarding pH in sputum, but NaCl was found to be higher in CF patients (10.5 in CF x 7.4 g/L in the control, respectively $p<0.05$) (Grandjean Lapierre *et al.*, 2017).

Many controversies remain, and thus it is extremely important to solve this question to direct an efficient therapy for patients, as in both conditions (lowered pH, or increased salt concentration), there are specific causes that can drastically change the strategies needed for treatment.

4.3.2. Hypertonic Saline (HS) as a therapeutic intervention

Hypertonic saline is a therapy used for a long time that is efficient and contributes to the mucocilliary clearance in CF. Some of the advantages related to its use is the cost, as it is not expensive, and can be applied by nebulization (Williams *et al.*, 2010). Molecular mechanisms described to be active after inhalation of this solution varies: the action of HS in ASL can be related to partial ASL hydration levels gained; it can break ionic bounds in the mucus, and release negative charge, alleviating viscosity; improve expiration that is consequently associated to reduction of inflammation and edema (Nenna *et al.*, 2017). It is used during long term maintenance therapy or in periods patients experience exacerbations. Concentrations of this solution more frequently used can vary from 3- 7% (Wark and Mcdonald, 2018).

Clinical trials using hypertonic saline have proven to be useful for CF patients. For example, in a double-blinded, randomized crossover study conducted with 12 young children with stable CF showing clinical signs, the effect of 7% HS was analysed. It was administrated twice a day for 6 weeks and an improvement of airway performance was observed (Nenna *et al.*, 2017).

This study agrees with other investigations in adults (Elkins *et al.*, 2006; Wark and Mcdonald, 2018). Another study indicates that response to treatment is dose-dependent for up to 12% HS (Robinson *et al.*, 1997). In CF patients treated with HS, symptom severity is greatly reduced and benefits are described to be related to discharge in sleep, congestion and dyspnea. HS reduces symptoms associated to CF and no serious collateral effects are detected (Dentice *et al.*, 2016).

Another important point that might be considered is that CF patients treated with HS temporally experience higher NaCl levels in the lungs. During CF, levels of NaCl were

described to be around 0.15M (Smith *et al.*, 1996), and hypothesized to be between 0.5 and 1.2M in CF lungs after treatment with HS (Williams *et al.*, 2010).

In a recent prospective study, with 57 patients and 62 controls, it has been shown that the concentration of salt in the sputum from CF patients treated with HS in CF was higher (0.2M) than in non-CF patients (or 0.13M, in control) (Grandjean Lapierre *et al.*, 2017). However, pre-treatment with a sodium channel blocker called amilore, did not show better outcome when used in combination with HS, as observed in clinical trials. Instead, patients pre-exposed to a placebo before hypertonic saline had better respiratory functions (Burrows *et al.*, 2014).

As seen during moments of worsening of CF infections, transient modifications in the levels of NaCl can occur by the continuous use of hypertonic saline. During these periods, microorganisms might encounter osmotic stress that is why we chose to cultivate bacteria in this condition to check if it could affect pathogenesis.

4.4. N-acetyl cysteine (NAC)

Changes due to the inflammatory reactions associated with the respiratory tract of CF patients are responsible for the severe lung damage associated with the disease. Typically, removal of adherent and dense mucus from these patients depends on physical mechanisms, such as physiotherapy, and / or chemicals, which can be administered orally or through inhalator therapy (Warnock and Gates, 2015).

N-acetyl cysteine is a substance used in clinical therapy for more than 50 years for the treatment of respiratory diseases. Its chemical formula is $C_5H_9NO_3S$ and it has a molecular weight of 163.2 g/mol (Ziment, 1988). It consists of an amino acid L-cysteine associated with an N-acetyl derivative, in addition to having a sulfhydryl group, which is responsible for its biological effects.

NAC can be obtained in powdered form, as tablets, syrups, and in parenteral presentations, being administered orally, intramuscularly, intravenously and / or by inhalation. Usually, oral administration is more common, but only little of this substance is able to reach tissues and plasma; the greater part (about 98%) is absorbed and incorporated into growing

protein peptide chains. Through this route, the peak plasma concentration is reached 1h after the administration, and its plasma half-life lasts around 2h; no trace of this substance is detected 10-12 h after administration. Clinically, it is considered a safe therapeutic agent and without major side effects (Kelly, 1998; Thorne Research, 2002).

NAC is considered an indirect antioxidant used as source of thiol and a Glutathione (GSH) precursor, participating in glutathione synthesis and peroxidase activity and detoxification. It also has direct action on oxidant radicals as a superoxide scavenger and is able to interact with ROS (Zafarullah *et al.*, 2003). It is also mucolytic agent used targeting mucin, the major component of mucus. It works through the interaction of its sulfhydryl group with disulfide bonds present in this mucoprotein (Zafarullah *et al.*, 2003).

The treatment with this substance causes depolymerization of the mucin and makes the mucus less viscous (Ehre *et al.*, 2014). Moreover, it is used as an expectorant agent, as it stimulates the ciliary action and the vagal gastro-pulmonary reflex, removing mucus from the upper airways (Ziment, 1988). In diseases characterized by a large accumulation of mucus in the lungs, such as in CF, NAC can be used, and its effect combined with antibiotics, usually leads to good results (Hussain *et al.*, 2015).

In addition to its anti-oxidative and fluidizing effects, NAC modulates the functioning of the immune system in CF patients. The presence of the modified CFTR receptor in upper epithelial cells, is related to an increase in the levels of interleukin 8 (IL-8), an important chemokine that acts in the recruitment of neutrophils to this region, potentiating tissue damage caused by the persistent presence of these cells. In addition, an imbalance of the redox potential is also observed in these patients, accompanied by pulmonary and intestinal oxidative disorders (Tirouvanziam *et al.*, 2006).

These alterations prevent intestinal absorption of naturally occurring antioxidants through food, and interfere with the secretion of glutathione, the endogenous metabolic antioxidant produced in the cytoplasm of cells. Studies conducted using a daily treatment with oral NAC in high doses for a short period of time (up to 1 month), demonstrate efficacy in the modulation of redox and inflammatory aspects in CF, and consequently, reduced IL-8 levels in sputum (Tirouvanziam *et al.*, 2006).

Besides its importance as a mucolytic agent, NAC is also known for its antimicrobial and antibiofilm properties against many pathogens (Olofsson *et al*., 2003; Zhao and Liu, 2010; Moon *et al*., 2015; Abdel-Baky *et al*., 2017; Pollini *et al.*, 2018), with reported effect on respiratory tract infections (Dinicola *et al.*, 2014; Blasi *et al.*, 2016). However, studies

specifically addressing CF infections are sparse, and when present, provide controversial clinical data. Despite all highlighted benefits and clinical evidence of its success to stabilize lung function (Dauletbaev *et al.*, 2009; Conrad *et al.*, 2015), some clinical trials show that NAC has no effect (Dauletbaev *et al.*, 2009) or report the absence of clinical benefits with its use as a therapeutic agent related to lung disease (Nash *et al.*, 2009).

The use of NAC is recommended in other circumstances. In acetaminophen / acetaminophen poisoning, the excess of these substances is related to the reduction of glutathione (GSH), an important antioxidant in the body (North *et al.*, 2010). In these cases, NAC acts as a donor of sulfhydryl for glutathione, restoring the plasma levels of this substance, and consequently, promoting the necessary detoxification of the organism. In other diseases characterized by oxidative damage, such as cancer, heart disease, and infectious diseases (for instance, caused by the human immunodeficiency virus, HIV) there is also a recommendation for its use (Thorne Research, 2002; Xiao *et al.*, 2016).

4.5. Cysteamine

Cysteamine (formula $HSCH_2CH_2NH_2$) is an amino thiol degradation product of the amino acid cysteine. This compound is naturally produced in humans and is derived from Coenzyme A pathway (Besouw *et al.*, 2013). It acts as precursor of the neurotransmissor taurine. In this case, natural levels of cysteamine are difficult to detect in the plasma as they are lower (<0.1 µM) than the detection limit (Besouw *et al.*, 2013).

This substance is used as a strategy of treatment for nephropathic cystinosis, a disease characterized by a storage lysosomal disorder in which cysteine is accumulated. For almost half a century, it has been prescribed to delay the progress of this genetic disease (Thoene *et al.*, 1976), and this is still the only drug in use. Cysteamine reaches the lysosomes by a non-identified transporter, and breaks the cystine found inside the lysosomes (Thoene *et al.*, 1976).

Then, cysteine, a cystine-derivative, is released by itself or combined with cysteamine as a disulfide cysteamine-cysteine (Besouw *et al.*, 2013). This way, both derivatives gain access to the cytoplasm by specific transporters. Cysteamine, then prevents accumulation of cystine inside lysosomes, turning it available in the cytoplasm (Besouw *et al.*, 2013). Although its

effectiveness in diminishing intralysossomal levels of cystine, renal system failure and Fanconi syndrome, that represent possible outcomes of cystinosis, cannot be avoided by its use (Elmonem *et al.*, 2017).

This substance can be easily found in many formulas. Cysteamine hydrochloride (C2H7NS·HCl), phosphocysteamine ($C_2H_7NS \cdot PO_3$), cysteamine bitartrate ($C_2H_7NS \cdot C_4H_6O_6$), or as a derivative of cystamine ($C_4H_{12}N_2S_2$), a bisulfide that undergoes reduction to cysteamine *in vivo* (Besouw *et al.*, 2013; Jeitner *et al.*, 2018) are some of the most common examples. Plasmatic levels of 0.03–0.07 mM can be reached from cysteamine bitartrate (15mg/kg) in humans (Smolin *et al.*, 1988).

Cysteamine is an FDA approved drug for over 20 years and a lot of expertise regarding its effects is available. This is one of the reasons it is really interesting to take the advantage of available data and safety associated to this substance. It is used for nephropathic cystinosis, but it has been investigated to treat other diseases, such as Huntington's disease (and other neurodegenerative disorders), nonalcoholic fatty liver disease, malaria, cancer, and, most recently, CF (Besouw *et al.*, 2013).

As cystinosis is an intracellular disorder, much of the knowledge related to its substance was gained due to progress in this field, and possible new applications have started exploiting its intracellular effects, as they are already available. In healthy individuals, cysteamine is described to act as a competition inhibitor for transglutaminase, inactivating this enzyme. In CF, transglutaminase is detected in high levels, and this is a consequence of the continuous production of ROS by neutrophils during infection of CF lungs, which are stimulated by the compromised clearance of pathogens from this site. As this enzyme is considered essential for autophagy (Jeitner *et al.*, 2018), this process is compromised in CF patients. Shrestha *et al.* investigated the effect of cysteamine in CF defective macrophages, where they found this substance improves autophagy of macrophages and has a direct effect against multi-resistant *S. aureus* (MRSA), *B. cenocepacia*, *B. multivorans* and *P. aeruginosa* (Shrestha *et al*., 2017).

5. Zebrafish as a model for virulence

Currently, virulence is defined as "the relative capacity of a microorganism to cause damage in a host" (Casadevall and Pirofski, 2003). Based on this, the use of *in vivo* systems are essential to analyse if a pathogen can cause disease (Washington, 1983). *In vitro* cell culture assays are important in the context of infection studies and have largely contributed to our understanding of host-pathogen interactions at the cellular level. However, animal models are essential to study infection mechanisms and analyse virulence and host-pathogen interactions as well as the host's immune response in the context of a living host (Casadevall, 2017).

Zebrafish (*Danio rerio*) is a small tropical freshwater fish with its natural habitat in India. Since the 1970's zebrafish have been used for developmental studies and about 20 years ago, it started to be used as a model for immunology, toxicology, and human diseases. For example, fish can be used to detect toxic substances in the water or in environmental samples (Hill *et al.*, 2005) and in drug screenings (Macrae and Peterson, 2015).

Zebrafish was also introduced as a model for human derived pathologies (Van Der Sar *et al.*, 2004), including cancer, hereditary and congenital diseases, inflammation, infection and immunological response (Lieschke and Currie, 2007). Its success as a model for infectious diseases can be seen from the large number of infection models that have been established since the early 2000s (Davis *et al.*, 2002; Van Der Sar *et al.*, 2003; Vojtech *et al.*, 2009; Vergunst *et al.*, 2010).

Several developments in the zebrafish research field have contributed to the great success of this small vertebrate as a biomedical research model. Researchers have been describing stages of embryonic development, which helped to better understand zebrafish life cycle (Kimmel *et al.*, 1995). The publication of its genomic sequence not only enabled genetic comparisons against the human reference genome, but it also allowed the introduction of omics and high throughput techniques, including proteomics and transcriptomics, to for instance, study the changes in host transcriptome during infection (Ruzicka *et al.*, 2019).

Although new features keep being discovered from ongoing annotation projects (Ruzicka *et al.*, 2019), available data show a high rate of similarities between the zebrafish and human genomes: about 70% of genes of humans were found to have orthologues in zebrafish, which makes zebrafish a good model to be employed to study human diseases (Howe *et al.*, 2013).

Various organs and cell types similar to humans are found in zebrafish (Kinth *et al.*, 2013), which is corroborated by the high genetic similarity.

The zebrafish immune system is also similar to that of humans. Although clear differences exist, major types of immune cells (macrophages, neutrophils, B-cells, T-cells, etc.) are present, as well as major immune signaling proteins such as tumor necrosis factor -alfa (TNF-alfa), IL1-alfa, IL-1ß, IL-6 and IL-8) and receptors (TLR-2, TLR-3, TLR-4 and TLR-5, for example), and for instance acute phase proteins, as Serum amyloid A, haptoglobin and hepcidin (Lin *et al.*, 2007; Van Der Vaart *et al.*, 2012).

An adaptive immune system is present between 2 to 4 weeks of life, which means the embryos and young larvae are attractive to study the host innate immune response during infections. Major cells of the innate immune system, macrophages and neutrophils, develop early in the life of the zebrafish: at 28 hours post fertilization (hpf) macrophages that can engulf and degrade bacteria are already circulating. Neutrophils can phagocytoses microbes from 30 hpf, while they are fully matured, containing granulocytes, at 2 days post fertilization (dpf) (Herbomel *et al.*, 1999; Van Der Vaart *et al.*, 2012) .

Other characteristics that have contributed to the popularity of the zebrafish embryo model include cheap maintenance and easy acquisition of eggs, easy manipulation and external embryonic development (Dahm and Geisler, 2006). Zebrafish also have a fast-embryonic development and high fecundity rate. Almost all organs are completely developed at 5 days post-fertilization. One adult couple can lay between 50- 100 eggs at a time, and using different couples, eggs can be obtained every day of the week (Hill *et al.*, 2005; Lieschke and Currie, 2007).

A major asset of the embryo and young larvae is their optical transparency, which allows detailed microscopic analysis in real time. This has given a major impulse to infectious disease studies, because in a live animal, in the context of an innate immune system, the interaction between microbes (viruses, bacteria, parasites) and host immune cells can be studied using fluorescent bacteria and transgenic reporter fish expressing fluorescent marker in specific cell types (Torraca and Mostowy, 2018).

In infectious disease studies, zebrafish has now taken an important place. In these infection studies, mainly using embryos, different sites can be used to introduce bacteria by injection, as shown in Figure 4. Different injection sites can be exploited, for example, to study zebrafish systemic effects, by injecting bacteria into the circulation, or as a model of bone and cartilage inflammation, when injection into the notochord is used (Torraca and Mostowy, 2018).

Models for infection with mycobacteria (*Mycobacterium marinum*, *Mycobacterium abscessus*, and *Mycobacterium leprae)*, Gram-positive (*Listeria monocytogenes* and *S. aureus)* and gram-negative bacteria (*Salmonella* Typhimurium, *Shigella flexneri*) have been developed (Torraca and Mostowy, 2018).

Vergunst *et al*. (2010) developed a model to study infections caused by bacteria belonging to the Bcc. Low doses of highly virulent clinical isolates, including *B. cenocepacia* K56-2, were able to kill embryos within three days post infection (Vergunst *et al.*, 2010). However, other strains as *B. stabilis* LMG12494 and *B. vietnamiensis* FC441/ LMG18836 could not establish a virulent infection (Vergunst *et al.*, 2010).

The innate immune system of the embryos could control the infection; however, bacteria could still survive and replicate intracellularly resulting in persistent, non-proinflammatory infection. Using this model, researchers in the Vergunst lab were able to demonstrate the key role of macrophages in the development of acute infection of *B. cenocepacia* in zebrafish (Mesureur *et al.*, 2017). An important contribution of these host immune cells was in line with a previous clinical study developed by Schwab and co-workers, as described above (Schwab *et al.*, 2014).

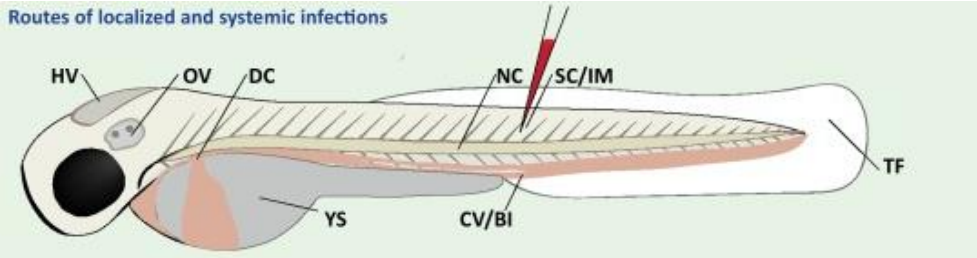


Figure 4 Routes of zebrafish injection.
Larvae can be injected locally into the YS or in body cavities, such as the HV and OV. Other compartments for injection include SC, IM, or the NC. HV, OV, IM, and TF infection all permit study of immune cell recruitment. The NC is inaccessible to immune cells but is valuable to model bone and cartilage inflammation. Injection into the circulation can be achieved by intravenous injections, for example via the CV/BI or the DC. This results in a rapid systemic dissemination of microbes throughout the body. Abbreviations: BI, blood island; CV, caudal vein; DC, duct of Cuvier; HV, hindbrain ventricle; IM, intramuscular; NC, notochord; OV, optic vesicle; SC, subcutaneous; TF, tail fin; YS, yolk sac. Available at https://doi.org/10.1016/j.tcb.2017.10.002.Source: Adapted from (Torraca and Mostowy, 2018)

BRIEF CONSIDERATIONS

Experiments are divided in the following two chapters (1 and 2). Chapter 1 describes all experiments done in Brazil, which include *in vitro* assays that were supervised by Prof. Eidy Santos and supported by CNPq. In chapter 2, we describe experiments involving zebrafish, that were done in collaboration and under the supervision of Prof. Annette Vergunst. The experiments described in chapter 2 were supported by Université de Montpellier (region of Languedoc Roussillion) and Institut National de la Santé et de la Recherche Médicale INSERM, and all of them were executed in France. A flowchart summarizing the experiments is presented in Appendix 1.

For most of *in vitro* assays done in Brazil, we used facilities of Laboratório de Bacteriologia e Bioensaios, located at Instituto Nacional de Doenças Infecciosas Evandro Chagas, at Fundaçao Oswaldo Cruz (INI/FIOCRUZ). For quality control purposes, classical biochemical tests with J2315 were done to confirm biochemical profile of the *B. cenocepacia.* This strain was kindly donated by Laboratório de Bacteriologia, that belongs to the Faculdade de Medicina which is affiliated with Universidade Estadual do Rio de Janeiro (UERJ),.

Growth curves, biofilm assays, adhesion to mucin and competitive assays, as well as cultivation for proteomic analysis were also done at FIOCRUZ. Still in Brazil, at Laboratório de Bioquímica located in Universidade Estadual da Zona Oeste (UEZO), culture supernatants of ET12 were extracted and purified. We used all facilities of Plataforma de Espectrometria de Massas, that belongs to Divisão de Metrologia Aplicada às Ciências da Vida (DIMAV) at Instituto Nacional de Metrologia, Qualidade e Tecnologia (INMETRO), for proteomic analysis.

The second part of this thesis that is related to zebrafish was done in France, in INSERM U1047, Virulence Bactérienne et Maladies Infectieuses, Université de Montpellier. Experiments included zebrafish infections assays, embryo survival, determination of bacterial burden, fluorescence microscopy and qRT-PCR analysis. In that laboratory, ASMDM media and effects of Cysteamine were analysed. Some complementary *in vitro* assays related to zebrafish assays, as bacterial fluorescence and growth curves for *B. cenocepacia* K56-2 and *B. stabilis* LMG12494, were also done in the same laboratory.

A scheme with a didactic overview of experiments done is shown as Appendix 2.

CHAPTER 1

In vitro analysis of *B. cenocepacia* virulence factors in abiotic systems

1. ABSTRACT

Many virulence factors are described in *B. cenocepacia*, including biofilms and adhesion to mucin. These abilities can be used to predict its virulence in the host through *in vitro* assays. Few information is available regarding the behavior of this bacteria in presence of conditions used to alleviate CF symptoms, such as hypertonic saline and NAC, that have been proposed to improve airway performance and to be mucolytic and expectorant agents, respectively. Based on this, we investigated the effect of both compounds in the *B. cenocepacia* virulence factors production. In this chapter, *B. cenocepacia* J2315 was cultivated in LB with NaCl (0.4M) + NAC (1g/L), NaCl at 0.1M, 0.3M, 0.4M and 0.5M and NAC (0.5, 1.0 and 2.0 g/ L). Bacteria was assessed for biofilm production, mucin adhesion, and in competitive assays against *P. aeruginosa* and *S. aureus*. In most tests, we analysed virulence factors at different growth phases (mid- or late-logarithmic, and stationary phase). NaCl at 0.4M and 0.5M and NAC at 2.0g/L were found to reduce the growth ratio of *B. cenocepacia*. Adherence to mucin (at stationary phase) and biofilms ($p \leq 0.01$) were higher with both conditions combined (LB 0.4M NaCl + 1g/L NaC). In general, more *B. cenocepacia* virulence factor production occurred during stationary phase. *B. cenocepacia* showed reduced ability to adhere to mucin and to produce biofilm in comparison with other CF -related pathogens, as *S. aureus* and *P. aeruginosa*. When previously adhered to mucin, *B. cenocepacia* was found to efficiently exclude *S. aureus* and *P. aeruginosa*. The *B. cenocepacia* ability to displace *P. aeruginosa* has completely changed from low (about 15%, during mid- log) to a high ratio (94%, at stationary phase), indicating a significant effect of the growth phase on the displacement of *P. aeruginosa*. In the exoproteome analysis, 38 proteins were detected in LB medium and 28 in LB 0.4M NaCl. From these 62 proteins, 9% were found to have unknown or hypothetical function, and only BipC, a predicted type 3 secretion system effector protein, was secreted in LB standard at 18h and 28h, but not in 0.4M NaCl as previously hypothesized by us. Our preliminary data suggest that *B. cenocepacia* may induce virulence factor under LB conditions, disclosed by the secretion of biofilm and the type 3 secretion system, but this phenotype seems to be inhibited at high salt concentrations without the presence of NAC.

Keywords: virulence factor production; NaCl; NAC; biofilm; mucin adhesion; competition

2. OBJECTIVES

2.1 General

- To evaluate in *B. cenocepacia* J2315 virulence factor production in presence of similar conditions related to CF lungs, as (i) higher concentrations of NaCl, to understand possible effects of hypertonic saline during chronic stages of CF lung infection; (ii) the presence of NAC, to simulate the effect of this mucolytic agent and to better understand possible interference with virulence factor production; (iii) high osmolarity associated to NAC.

2.2 Specifics

- To define the growth phases of *B. cenocepacia* J2315 in LB

- To evaluate if NaCl (0.1, 0.3, 0.4 and 0.5M) and NAC (0.5, 1.0 and 2g/L) affect *B. cenocepacia* growth

- To evaluate biofilm production by *B. cenocepacia, S. aureus* and *P. aeruginosa* in presence of 0.4M NaCl, 1g/L NAC or 0.4M NaCl + 1g/L NAC at 24h (*S. aureus* and *P. aeruginosa*) or after 18 and 28h (*B. cenocepacia*) of growth.

- To analyse *B. cenocepacia, S. aureus* and *P. aeruginosa* mucin adhesion in the same conditions described in the previous topic.

- To study the ability *B. cenocepacia* to exclude and displace *S. aureus* and *P. aeruginosa* by competitive inhibition during mid-LOG and stationary growth phase.

- To look specifically for secreted virulence factor differently expressed in LB and LB supplemented with NaCl (0.4M), after 18h, 24h and 28h of growth at 18h, 24h, 28h by means of proteomic analysis

3. MATERIAL AND METHODS

3.1. Bacterial strains, quality control and stocks

B. cenocepacia J2315 is a highly transmissible epidemic clone that was isolated for the first time in Edinburgh, in the United Kingdom (ET-12) (Govan *et al.*, 1993). It was kindly provided by Dr. Elizabeth de Andrade Marques (Department of Microbiology, Immunology and Parasitology of Universidade do Estado do Rio de Janeiro- UERJ). *S. aureus* American Type Culture Collection (ATCC) 25923 *and P. aeruginosa* ATCC 27853 were used in this study as well.

Stocks were prepared from pure colonies obtained on 5% sheep blood agar grown at 36 ± 1 ° C for 24 to 48 hours. They were kept cryopreserved on Tripticasein Soy Broth (TSB) with glycerol (15%) at -20 ± 1 °C. *B. cenocepacia* J2315, *S. aureus* ATCC 25923 *and P. aeruginosa* ATCC 2785 were used to access biofilm production, adhesion to mucin, and on inhibition assays. Also, we used *B. cenocepacia* J2315 to obtain growth curves and exoproteomes. All strains had previously been identified based on molecular tests and checked to accomplish with standard biochemical and physiological tests expected for their profile (Jorgensen and Pfaller, 2015; Clsi, 2019).

To confirm the biochemical profile of the strain, we used a panels for gram-negative microorganisms, and the following tests were performed: Sulfate/Indol/Motility (SIM), Double sugar urea (DAU), Citrate, pyrrolidonyl aminopeptidase (PYR) Hydrolysis Test, Motility with 2,3,5-Triphenyltetrazolium Chloride (TTC), oxidative / fermentative (O/F), Indol Test, deoxyribonucleic acid (DNA)se, Sulfamethoxazole Susceptibility Tests + Trimethoprim (SXT), Meropenem (MEM), Ceftazidine (CAZ) and Minocycline (MH).

We also carried out specific tests for *B. cenocepacia complex*, such as Oxidase, Growth at 42 + 1ºC and MacConkey agar, pigment, Lysine and Ornithine production, Decarboxylases, Esculin Hydrolase and Susceptibility to Polymyxin B (Jorgensen and Pfaller, 2015). Reference strains of the ATCC were used for validation of antimicrobial discs, culture media and biochemical tests according to the instructions described by the

manufacturers and / or established in the literature (Jorgensen and Pfaller, 2015; Clsi, 2019).

In addition to traditional phenotypic methods, automated identification was also used, with a specific card containing biochemical tests for gram-negative bacilli. A volume of 3 mL of a suspension equivalent to 0.5-0.63 scale in nephelometer was transferred to Gram-negative (GN) ID card and analysed with Vitek II (bioMèrieux) following manufacturer's recommendations. This experiment was carried out in triplicate.

3.2. Growth conditions and culture media

For cultivation of bacteria, strains were routinely obtained from cryotubes. They were pre-activated in LB Vegitone broth and incubated at 36 ± 1 °C with aeration (9 x g, 24-48h). After bacterial growth, suspensions were centrifuged (27.000 x g for 5 min), and pelleted cells were washed and resuspended in saline solution (0.9%). A nephelometer was used to obtain cell density of 0.5 in McFarland scale.

Then, we diluted the suspensions to a concentration of approximately 10^7 CFU/mL which was used in specific media. For experiments, growth was performed at the same temperatures but, instead of aeration, static cultures were used. At first, we used an overnight culture to grow bacteria for the growth curve. After, the incubation time was adjusted in each experiment according to the bacterial growth phases.

As stated in Table 1, LB Equitone Broth was complemented with (i) NaCl to obtain LB 0.1M, 0.3M, 0.4M and 0.5M NaCl, with (ii) NAC to obtain 0,5 g/L, 1,0g/L or 2,0g/L NAC, or (iii) with NaCl and NAC to obtain LB 0.4M NaCl and 1,0g/L NAC. NAC was prepared immediately before use, filtrated in 0.22µm membranes, and added to the autoclaved medias to obtain LB + NAC at 0.5 g/L, 1.0 g/L and 2.0 g/L.

Table 1 Culture media used for *B. cenocepacia* J2315

Culture Media	**(C_{final})**
LB	
Tryptone	10g/L
Yeast Extract	5g/L
NaCl	5g/L
LB + NaCl	
LB 0.1M NaCl	5.9 g/L
LB 0.3M NaCl	17.6 g/L
LB 0.4M NaCl	23.4 g/L
LB 0.5M NaCl	29.2g/L
LB + NAC	
LB 0.5 NAC	0.5 g/L
LB 1.0 NAC	1.0 g/L
LB 2.0 NAC	2.0 g/L
LB + NaCl + NAC	
LB 0.4 NaCl 1 NAC	0.4M + 1.0g/L

3.3. Growth curve

To obtain growth curves, we started with an inocula with 10^7 CFU/ mL in LB. Sterile 96-well polystyrene plates with flat bottom were used to obtain the OD_{600}. Flasks with the bacterial culture were kept without aeration in the incubator for 48h at 36 $\pm$1ºC. At first, *B. cenocepacia* J2315 growth phases were defined based on OD_{600} and CFU counting at the time points 0h, 5h, 10h, 20h, 25h, 30h, 35h, 45h and 48h. CFUs were expressed in $\log_{10}$ (CFU/mL) in one experiment performed.

After, we tested LB with NAC and NaCl, in all conditions described in the previous section to check if complementing LB with increasing concentrations of NaCl and NAC it could affect bacterial growth. We started with 10^7 CFU/ mL, and we used the OD_{600} to compare the bacterial growth. Non-inoculated LB (or LB complemented with different conditions) was used as a negative control.

Microplates were incubated in the microplate reader for 22h at 36 ± 1ºC, and the equipment was programmed for readings every hour, with prior shaking of 15s. Each condition was performed at least in triplicates in individual experiments, and it was repeated once. The results expressed are means obtained in one representative experiment.

3.4. *In vitro biofilm production*

To assess the *B. cenocepacia* ability to produce biofilm, sterile 96-well polystyrene plates with flat bottom were used. We started from 10^7 CFU/ mL suspensions, and 200µL were delivered into each well. Microplates were incubated at 36 ± 1° C, without shaking. Culture media used for biofilm detection were LB, LB 0.4M NaCl, LB with 1.0 g/L NAC; LB 0.4M NaCl + 1g/L NAC. *B. cenocepacia* was evaluated during mid-log and stationary phase. For comparisons, *S. aureus* and *P. aeruginosa* were grew for 24h on the same culture media, as these species are known as biofilm-producers related to CF.

The ability of *B. cenocepacia* to produce biofilm was quantified according to Tendolkar and colleagues (Tendolkar *et al.*, 2004), with the following modifications. As we observed differences in bacterial growth in the modified culture media, we normalized the biofilm production based on the growth. After incubation, OD was measured at λ = 570nm in SpectraMax Plus 384 Microplate Reader (Molecular Devices). At first, OD was obtained before removal of the planktonic cells, to assess growth, previous to the staining of the microplates with violet crystal. After staining, individual tips were used to homogenize biofilms residues stained onto the walls of each single well, and then, we obtained a second OD_{570} value after the treatment with violet crystal, to measure biofilm produced. To normalize biofilm production, we divided final OD obtained after staining, indicative of biofilm production, by the OD registered before staining (bacterial growth). The final values obtained were used to construct the dot plot. Non-inoculated wells containing LB broth were used as a control. Each assay was performed twice, with seven biological replicates. Representative results of one of the experiments are presented in Figure 6 (O'toole and Kolter, 1998).

3.5. *In vitro adhesion to mucin*

The procedure described by Valeriano and co-workers (Valeriano *et al.*, 2014) was used to assess adhesion of *B. cenocepacia* J2315 to mucin. To evaluate the ability to

adhere to mucin, *B. cenocepacia* was grown in LB, LB 0.4M NaCl, LB 1.0g/L NAC and LB 0.4M NaCl + 1.0g/L NAC. Some modifications indicated below were based on a previous work developed by Sanchez (Sanchez *et al.*, 2010). *B. cenocepacia* was grown for 18h (mid- logarithmic) and 28h (Stationary) phase to explore differences in adhesion referent to the growth phase. *S. aureus* and *P. aeruginosa* were included for comparisons with *B. cenocepacia*, as they are commonly associated to CF infections. In this case, these bacteria were grown for 24h and 28h to compare with *B. cenocepacia* in the mid-logarithmic and stationary phase, respectively.

Approximately 100µL of mucin from porcine stomach Type II partially purified (1mg/mL, Sigma- Aldrich ®), dissolved in phosphate-buffered saline 1x (PBS 1x), was added to each well of sterile 96-well plates. Polystyrene flat bottom microplates used were incubated for 1h at 36 $\pm$ 1° C. They were then let overnight to immobilize at 4° C. Wells were then washed twice with PBS 1x to remove non-bound mucin. Then, 100µL of bovine serum albumin (BSA at 20 mg/mL in water for injection) were used to block non-bound sites on the wells and incubated for an additional 2h at 4° C. After this, plates were carefully washed twice with 200µL of PBS 1x. Overnight cultures of bacterial cells at exponential (18h) or stationary (28h) phase were washed once with 0.9% saline solution, adjusted to 10^7 CFU/mL in PBS 1x and from this suspension, 100 µL were inoculated on each well containing mucin for 1h at 36 $\pm$ 1° C. It was followed by five washing steps with citrate buffer (200 µL), used to remove non-adherent bacteria. An additional volume of 200 µL of Triton X-100 solution at 0.5% (v/v) was used to detach bacteria from the wells, and viable bacterial counts were determined by plating in Mueller Hinton Agar (MHA), and expressed as CFU/mL. Percentage of adhesion was obtained according to Collado (Collado *et al.*, 2008) based on the formula described below. Two independent experiments were done, and graphs were constructed with data obtained in one representative experiment.

$$\% \text{ Relative Adhesion} = \frac{\text{CFU/ml after adhesion}}{\text{CFU/ml before adhesion}} \times 100$$

3.6. *In vitro exclusion and displacement assay*

To evaluate the behavior of *B. cenocepacia* J2315 in presence of *S. aureus* and *P. aeruginosa*, the ability of *B. cenocepacia* to exclude and displace these other species was investigated. Briefly, this assay was based on (Collado *et al.*, 2008), adapted (Valeriano *et al.*, 2014) and modified to include minor changes, as described here. LB Vegitone Broth was used to grow strains, at the same conditions described above for adhesion assay. Microplates were prepared as described in the previous session.

After wells were washed with PBS to remove non-bound BSA, *B. cenocepacia* J2315 was incubated to adhere to mucin, the same way described in the mucin adhesion assay. After 1h at 36 $\pm$ 1^{o} C, the suspension was completely removed, and wells were washed twice with PBS 1x. Then, a volume of 100 µL of the second suspension (containing *S. aureus* or *P. aeruginosa*), was inoculated and incubated at 36 $\pm$ 1^{o} C for additional 1h for the exclusion assay.

Non-adhered bacteria were removed by five cycles of washing with 0.9% NaCl. Each well was treated with 200 µL of Triton X-100 to detach adherent bacteria, and by the same way described before, CFU was obtained by plating. Inversely, for displacement assays, plates were first incubated with *S. aureus* or *P. aeruginosa*, and then with *B. cenocepacia*. Experimental quadruplicates were prepared for each condition, used to obtain the media and from this, we calculated percentage of exclusion and displacement. Each experiment was repeated once. Percentage of inhibition was calculated based on (Collado *et al.*, 2008), according to the following formula described below. Averages and standard deviation were calculated and used to express the results obtained.

$$\% \text{ Inhibition} = \frac{(\% \text{ adherence of pathogen}) - (\% \text{ adherence of pathogen with probiotic})}{\% \text{ adherence of pathogen}} \times 100$$

3.7. Exoproteome characterization

3.7.1. Preparation of extracellular proteins for proteome analysis

Exoproteomes were obtained from bacteria cultivated in LB and LB 0.4M NaCl, and characterized according to Santos and colleagues (Santos *et al.*, 2011). Extracellular proteins were extracted after 24h of growth (1st assay; late-log phase) or at 18h/28h (2nd assay; mid-log/stationary phase). Supernatants were recovered by centrifugation (8000 x g for 5 min) and separated from bacterial cells. Exoproteomes were kept at -20ºC until analysis. Two independent cultures for each condition were analysed.

Briefly, 20 mL of supernatants was filtrated (0.22 µm) to exclude bacterial cells. Exoproteomes were purified by precipitation with trichloroacetic acid (TCA, 100%). TCA was added to samples to a final concentration of 20% (v/v), followed by incubation on ice for 15 min, and centrifugation at 12,000 x g for 30 min at 10 °C. Supernatants were discarded, and pellet containing proteins were then washed with 5 mL of ice cold acetone, P.A.

Samples were vortexed and kept on ice for around 15 minutes. After incubation, they were centrifuged at 12,000 x g for 15 min and acetone was removed. Washing step with acetone was repeated twice. After discarding the supernatant, the pellet was suspended in 50mM Ammonia Bicarbonate.

Initially, for late-log experiments (24hs), proteins were suspended in 5 mL ammonia bicarbonate and then concentrated by using centrifuge tubes (0.5 ml) with Amicon Ultra type filter (Millipore) containing 3KDa cellulose membranes. From each 5 mL volume, 3.5 mL were separated to be processed; the remaining volume (1.5 mL) was stored in a freezer at -20ºC. Aliquots of approximately 450 µL were transferred to the centrifuge tubes and subjected to repeated cycles of 12,000 x g for 10 min, ensuring the passage of all material through the membrane. A volume of 150 µL of Ammonia Bicarbonate was used to solubilize.

Afterwards, mid-log and stationary exoproteomes were suspended in 300 uL of ammonia bicarbonate after TCA precipitation and the Amicon concentration step was not

performed, to improve protein extraction. Proteins were quantified by the Bradford method (Bradford, 1976), using a Shimadzu UV-VIS 2420 UV-VIS spectrophotometer.

3.7.2. Exoproteome identification by mass spectrometry (MS)

For each sample, total exoproteomes were characterized and analysed by mass spectrometry. The complex protein mixture was subjected to trypsin digestion in a shotgun methodology. After quantification, an aliquot containing 50 µg of secreted proteins was subjected to trypsin digestion. To each sample, 10 µL of 50 mM ammonium bicarbonate and 25 µL of 0.2% RAPIGEST SF were added.

The mixtures were incubated at 80 °C for 15 min. This was followed by the addition of 2.5 µL 100 mM DTT with incubation at 60 °C for 30 min. After cooling down the samples, iodoacetamide was added to 300 mM (final concentration) and samples were incubated in the dark for 30 min. Then, 10 µL of trypsin solution (Promega) (15 µg /µL) was added for digestion, which occurred at 37 °C for 16 h.

The next day 10µL of 5% trifluoroacetic acid (TFA) was added. The samples were then incubated at 37 ° C for 90 min. The samples were centrifuged at 12,000 x g at 4 ° C for 30 min and the supernatant transferred to a glass container compatible with the Waters vial. Mid-log and Stationary culture peptidomes were submitted to solid-phase extraction with the Oasis HLB cartridge (Waters Corporation, Milford, MA, USA) before the transference to glass vials. Finally, 5 µL ADH (1pmol/µl) and 85 µL 3% ACN and 0.1% formic acid were added. The final concentration of inserted peptides (from trypsin digestion) used in the MS analysis was 250 ng/µL and of ADH, was 25 fmol /µL in a final volume of 200 µL.

3.7.3. **Liquid Chromatography Coupled to Tandem Mass Spectrometry**

MS analysis was carried out on a nano ultra-performance liquid chromatography system (nanoACQUITY) coupled to a mass spectrometer Quadrupole-Time of Flight SYNAPT G1 (Waters), situated in INMETRO facilities, at Plataforma de Espectrometria de Massas. Tryptic peptides obtained were initially injected and desalted on a Trap Symmetry C18 column (5 µm particle size, Waters), for 3 minutes (flow rate of 5 µL/min). The mixture of trapped peptides was then separated by high performance liquid chromatography in a HSS T3 C18 column (100 Å 1.8 µm, Waters) heated to 55 °C and at a flow rate of 0.450 µL/min with 30 min of run total time. Mobile phases consisted of (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in 100% acetonitrile. We used as the following separation gradient: 7-to-40% (B) during 20 min, 40-to-85% (B) for 4 min, 85% (B) for 4 min and 85-to-7% (B) during 2 min.

Electrospray tandem mass spectra were performed in the positive detection ion mode, and instrument control and data acquisition were conducted by MassLynx data system (Version 4.0, Waters). Data were acquired in an expression mass spectrometry mode (MSE), and multiple charged peptides ions (+2 and +3 and +4) were automatically mass selected and dissociated in MS/MS experiments. Typical ESI conditions were 3 kV nanoflow capillary voltages and 35 V cone voltages. Three experimental replicates were processed and analysed individually.

3.7.4. **Protein identification and Data Analysis**

Protein identification was carried out using ProteinLynx Global Server v3.02 software (Waters). We submitted raw data against a database with reviewed and unreviewed protein sequences from *Burkholderia* genus obtained from UniProt Knowledgebase (UniProtKB; http://www.uniprot.org/uniprot/).

Analysis was performed based on the following parameters: minimal fragment ion matches per peptides, 3; minimal fragment ion matches per protein, 7; minimal peptide matches per protein, 1; trypsin missed cleavages, 1; carbamidomethylation of cysteines as a fixed modification and oxidation of methionine as a variable modification. Proteins were sorted as real identified when detected in at least two experimental replicates. Comparative expression analysis was performed using the Expression software v2.0 present in the package ProteinLynx Global Server.

Identified proteins were manually classified according to biological process using Gene Ontology database (Consortium, 2015). Direct (physical) and indirect (functional) protein-protein interaction were established using software STRING (Franceschini et al., 2013).

Comparative analyses of identifications were made using the Venn Diagram tool from the Bioinformatics & Evolutionary Genomics Platform (http://bioinformatics.psb.ugent.be/webtools/Venn/).

3.8. Statistical analysis

Statistical analysis was performed using R version 3.6.0 and charts were constructed using Prism Version 6. For growth curves, trend values with 95% confidence intervals were estimated by Ordinary Least Square (OLS) in order to compare them. The Wilcoxon Test was performed to evaluate the difference between biofilm production and mucin adhesion assay median.

The median value of individual bacterial species in each different culture media for biofilm assay and mucin adhesion were compared in relation to the control (LB). In *B. cenocepacia*, growth phases (mid- log and stationary) were also compared. Although not indicated in the graphics, different species (*B. cenocepacia* during mid-log and stationary phase, *P. aeruginosa* and *S. aureus*) were compared in each culture medium, statistically analysed and results obtained are described in each section.

Additionally, for mucin adhesion assay, both biological replicas were compared and only one representative experiment is shown. For *S. aureus* and *P. aeruginosa* inhibition

assays, averages of percentages obtained in two independent experiments + SD were showed.

4. RESULTS

4.1. Confirmation of purity and identity of *B. cenocepacia* J2315

Before starting experiments, we performed biochemical and physiological tests to confirm purity of *B. cenocepacia* received from other collection, as a default practice in the laboratory to keep quality control standards. Results obtained are described in Table 2. Bacteria were identified as Gram-negative bacilli and characterized as being positive oxidase, able to grow at 42 °C and in MacConkey agar, with colonies pigmented in salmon. Lysine and ornithine decarboxylases, as well esculin hydrolase activities were positive. Antimicrobial sensibility test revealed resistance to polymyxin B (6mm). Biochemical results obtained manually to screen non-fermenting gram-negative bacilli gave results compatible with the profile of *Burkholderia* sp.

Table 2 Biochemical profile of *B. cenocepacia* J2315

Classical tests	Results	Screening methods	Results
Gram	BGN	SIM	MOT+ / H_2S -
Oxidase	+	Urea Agar Test	+
Growth in MacConkey	+	Citrate	+
Growth at 42±1ºC	+	PYR	-
Yellow pigment	-	*Motility* + TTC	+ (*)
Lysine Decarboxylase	+	O/F	O:+ / F:-
Ornithine Decarboxylase	+	Indole	-
Esculin hydrolase	+	Polymyxin B (PB)	6mm (R)
DNAse	-	SXT	6mm (R)
		MEM	12mm (R)
		CAZ	18mm (I)
		MH	13mm (R)

+, positive; –, negative. Gram: Gram staining; BGN: Gram-negative bacilli; SIM: Sulfate/Indol/Motility; MOT: Motibility; PYR: L-pyrrolidonyl arylamidase; TTC: 2,3,5, tripheniltetrazolium chloride; O/F:

Oxidation/ Glucose fermentation; I: intermediary; R: resistant; SXT: Sulfamethoxazole + Trimethoprim; MEM: Meropenem; CAZ: Ceftazidime; MH: Minocycline; (*) Similar to the classical swarming motility expected for *P. aeruginosa.*

A Gram-negative card of Vitek 2 ® compact (BioMeriéux) automated system was used in parallel to confirm results. Through this methodology, results obtained varied between *Sphingomonas* sp. (99% probability) or *P. aeruginosa* (with 91% and 97% probability, identified after 7 consecutive repetitions, at 7.25h and 10.25h, respectively) in three analyses performed (data not shown). Results obtained by automated system were not able to confirm the profile obtained by manual methods. Traditionally, it is well accepted in Diagnostic Microbiology Laboratories (oral communication) that Vitek has no resolution to identify non fermentative Gram-negative bacilli, especially in the case of *B. cenocepacia*. Based on this, we decided not to consider this difference, and we followed experiments with the strain validated as *B. cenocepacia*.

4.2. At higher concentrations, NaCl and NAC slowed down *B. cenocepacia* J2315 growth kinetics

During treatment, NAC and HS can be transitorily present in the lungs of CF patients. To investigate virulence factor production by *B. cenocepacia,* we used LB complemented with NAC and NaCl to evaluate if the presence of these substances would impair *B. cenocepacia* growth. Based on CFU and OD_{600}, we obtained *B. cenocepacia* J2315 growth curves on LB (Figure 5a). Mid-log, late-log and stationary phase were reached at 18h, 24h and 28h, respectively (Figure 5a).

Absorbance at OD_{600} was also used to obtain growth curves in LB + X NaCl (X= 0.1M, 0.3M, 0.4M and 0.5M) and LB + Y NAC (Y= 0.5g/L, 1g/L or 2.0 g/L). Results showed on Figure 5b indicate that, any of the conditions tested exerted a complete inhibition against J2315 growth. At 0.1M NaCl, 0.5g/L and 1.0 g/L of NAC, bacterial growth was similar to LB.

Nevertheless, in LB + NaCl 0.3M, and more accentuated in LB 0.4M or 0.5M NaCl and in LB 2.0g/L NAC, growth was slower than the control with LB. Time trends of *B.*

cenocepacia J2315 growth obtained for each individual growth curve with a confidence interval of 95% (Table 3) confirmed differences showed in Figure 5b. In Figure 5b it is possible to see that bacteria usually take 6h to start growing in most of culture media tested. However, in presence of LB 0.4M or 0.5M NaCl and in LB 2.0g/L NAC, bacteria take more time to start log phase (11h) and have a less pronounced growth, expressed by means of OD.

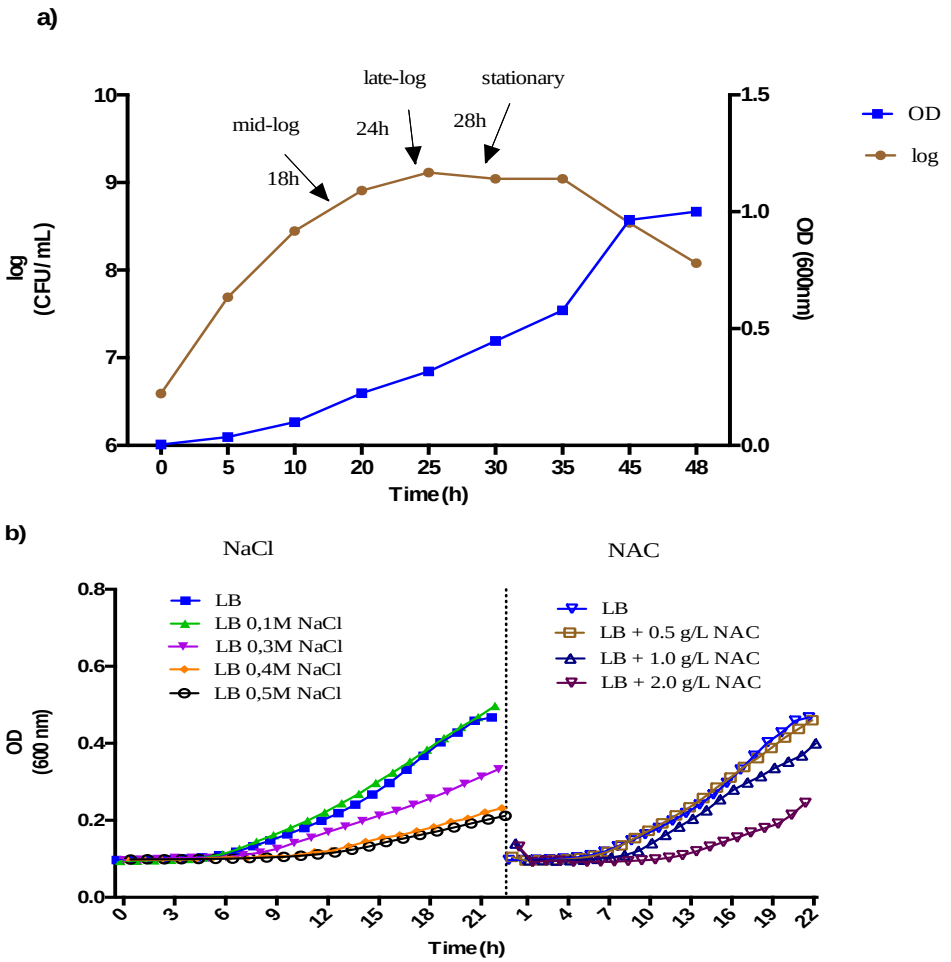


Figure 5 *B. cenocepacia* J2315 ability to grow in different culture media.
In (a), growth curve obtained in LB media based on OD (600nm) and log (CFU/ml). Mid- log (18h), late-log (24h) and stationary (28h) phases are indicated. In (b), bacteria were cultivated in different concentrations of NaCl (0.1M, 0.3M, 0.4M and 0.5M) and NAC (0.5, 1.0 and 2.0 g /L). Growth curves were obtained up to 22h and expressed as OD (600nm).

Table 3 Time trends of *B. cenocepacia* J2315 growth in different culture media.
Time trends were obtained for each culture media based on growth curves calculated (OD600). The 95% confidence intervals estimated by Ordinary Least Square (OLS) are also shown.

Culture Media	Time Trend (OD600)	CI Lower (95%)	CI Upper (95%)
LB	0.0181	0.0156	0.0207
LB 0.1M NaCl	0.0194	0.0171	0.0216
LB 0.3M NaCl	0.0111	0.0096	0.0125
LB 0.4M NaCl	0.0061	5	0.0071
LB 0.5M NaCl	0.0052	0.0042	0.0061
LB	0.0181	0.0156	0.0207
LB 0.5 g/L NAC	0.0178	0.0157	0.02
LB 1.0 g/L NAC	0.0144	12	0.0168
LB 2.0 g/L NAC	0.0069	0.0047	9

4.3. *B. cenocepacia* biofilm production increases at stationary phase and it is lower in presence of 0.4M NaCl or 1g/L NAC

We assessed the *B. cenocepacia* ability to produce biofilm in presence of NaCl and NAC at mid-log and stationary phase. For comparisons with *B. cenocepacia*, we grew *S. aureus* and *P. aeruginosa* in overnight cultures on the same culture media, as these species are biofilm-producers related to CF. As can be seen in Figure 6, the ability to produce biofilm of *P. aeruginosa* was the strongest, while *S. aureus* was moderate and *B. cenocepacia* was the lowest (Figure 4a, b and c). Across species, in all comparisons done between similar condition, for instance LB x LB, the p value was $p < 0.0001$.

The addition of 0.4M NaCl in the media used to cultivate bacteria negatively affected the *B.cenocepacia* (*B. cenocepacia*, $p < 0.001$, at mid-log phase) and *P. aeruginosa* ($p \leq 0.01$) biofilm production (Figure 6a, b). However, no difference was observed for *S. aureus* in comparison with the control ($p = 0.11$, Figure 6c).

In respect to *B. cenocepacia* growth phases, more biofilm was produced during stationary phase (Figure 6a). In all pair-pair comparisons, the significance obtained between growth phases with the same media was $p \leq 0.0001$ (Figure 6a). The only exception was verified when either LB 0.4M NaCl and NAC (1g/L) were added to LB,

as no difference in biofilm production was detected between *B. cenocepacia* mid-log and stationary phases (p = 0.42, Figure 6a). However, *B. cenocepacia* biofilm in presence of 0.4M NaCl and NAC (1g/L) showed greater biofilm levels on mid- log phase in relation to the LB used as control (p ≤ 0.01, Figure 6a).

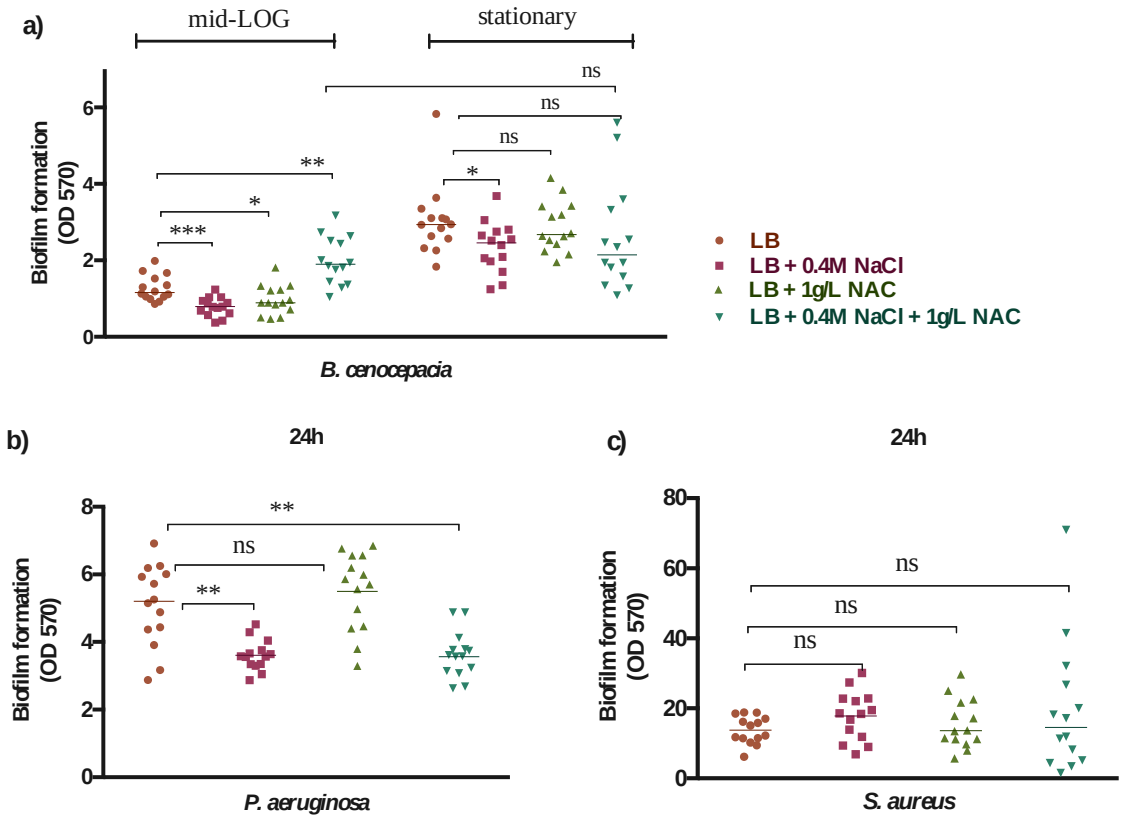


Figure 6 Biofilm production in different growth phases.
On the top, *B. cenocepacia* scatter charts in mid-logarithmic (mid-log, at 18h) and Stationary (28h) growth phases (a); on the bottom, *P. aeruginosa* (b) and *S. aureus* (c) scatter chart representing biofilm formation after 24h of growth. Each condition used to grow bacteria is represented in a vertical line, and each symbol represents individual values of experimental (n=7) and biological replicates (n=2). Horizontal bars represent medians obtained. Groups are represented as follows: LB medium (control, brown, dots) supplemented with 0.4M NaCl (pink, squares), 1.0 g/L NAC (light green, regular triangles), or both (0.4M NaCl + 1.0 g/L NAC, dark green, inverted triangles). Each well was inoculated with 200µL from a suspension containing approximately 10^7 CFUs/mL. Biofilms were revealed with crystal violet. On the y-axis, ratio of absorbances obtained before and after staining with violet crystal with readings at OD 570nm; on the x-, microorganisms are indicated. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001 and ns: not significant. See material and methods for statistical analyses.

4.4. The ability of *B. cenocepacia* to adhere to mucin increases during stationary phase and is reduced in presence of either 0.4M NaCl or 1g/L NAC

The adhesion to mucin was investigated here as another virulence factor produced by *B. cenocepacia*. We found *B. cenocepacia* had a higher adhesion to mucin during the stationary in comparison with mid-log phase (Figure 7a). Adhesion to mucin was lower in presence of either 0.4M NaCL or 1g/L NAC, except during stationary phase in which it was found to be equal to the control (LB). Only when these compounds were combined, an increase in the adhesion to mucin was observed (Figure 7a). *S. aureus* and *P. aeruginosa* showed the highest adhesion to mucin rates after 24h of growth in comparison with *B. cenocepacia* in both phases.

In Figure 7b, between *B. cenocepacia* mid-log and stationary phase, *P. aeruginosa* showed an expressive difference in its ability to adhere to mucin. We have used the same culture for both experiments, with only four hours of difference between experiments (Figure 7b). We suppose during the time -between processing (4h), *P. aeruginosa* reached a critical phase in which bacteria varied deeply in its ability to adhere to mucin. Although these differences showed on graphics, no statistical difference was observed, considering α=0.05, probably due to the limited sample size, and the consequent low statistical power of the analysis.

4.5. Inhibition of *S. aureus* and *P. aeruginosa* by *B. cenocepacia*

Results obtained in inhibitory assays are showed in Table 4. The exclusion assay measures how much a *B. cenocepacia* associated to mucin impairs the adherence of a 2nd pathogen (*S. aureus* or *P. aeruginosa*). In the displacement assay, the pathogens are already adhered to mucin when *B. cenocepacia* is included in the system. Then the *B. cenocepacia* ability to remove (or displace) pathogens previously adhered to mucin is measured.

B. cenocepacia has showed ability to exclude and displace *S. aureus* and *P. aeruginosa* strains at both growth phase (mid-log and stationary). *S*trong differences to displace *P. aeruginosa* according to the growth phases were detected: although displacement at mid- log was very low (14.99±2.55), it showed to be highly accentuated during stationary phase (93.48±0.37). In an opposite way, *B. cenocepacia* showed better ability to displace *S. aureus* in earlier steps, at mid- log phase (66.69±2.08), but it was kept during stationary phase (46.63±29.29). In the exclusion assays, *B. cenocepacia* showed similar ratios against both, *S. aureus* and *P. aeruginosa* strains, and among growth phases tested too. *B. cenocepacia* excluded *S. aureus* with 79.39±15.68 or 75.20±29.44 and *P. aeruginosa* with 91.92±0.66 or 96.4±0.29, during mid-LOG and stationary phases, respectively.

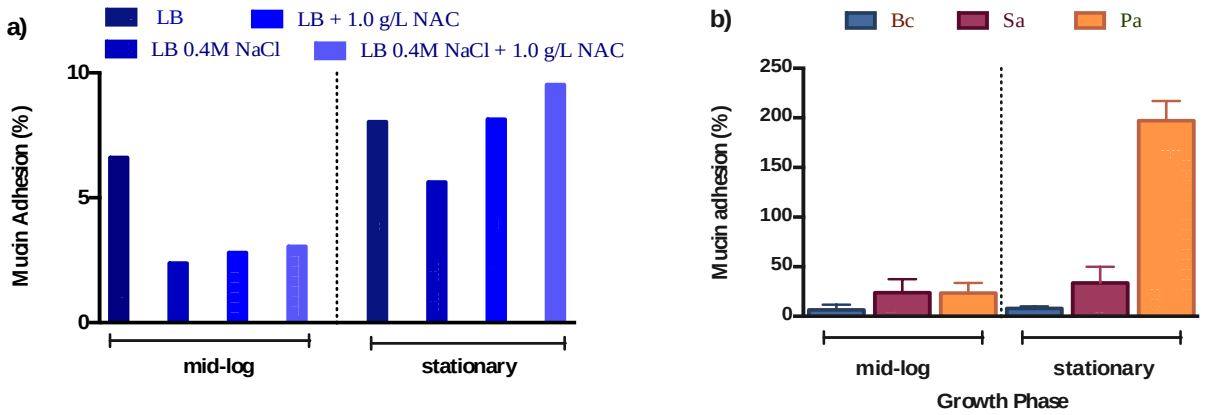


Figure 7 *B. cenocepacia* adhesion values (%) to mucin in mid-logarithmic (mid-log) and stationary phase of growth.
Per cent values were calculated from CFU obtained before and after adhesion. In (a), adhesion after growth in LB (control), LB 0.4M NaCL (with or without + NAC at 1g/L) and LB with NAC (1.0 g/L). In Figure (b), *B. cenocepacia* (Bc) adhesion to mucin in comparison with *S. aureus* ATCC25923 (Sa) and *P. aeruginosa* ATCC27853 (Pa). This experiment was performed twice and representative results are shown. Statistical analysis were done with Wilcox Test for two independent samples.

Table 4 Inhibition Assay of *S. aureus* and *P. aeruginosa*.
Relative adhesion of *S. aureus* (ATCC25923) and *P. aeruginosa* (ATCC27853) caused by *B. cenocepacia*. Averages of percentages obtained in two independent experiments + SD were showed.

	mid-logarithimic phase			
	S. aureus (ATCC25923)		*P. aeruginosa* (ATCC27853)	
	Exclusion	Displacement	Exclusion	Displacement
B. cenocepacia ET12	79.39 ± 15.68	66.69 ± 2.08	91.92 ± 0.66	14.99 ± 2.55
	stationary phase			
	S. aureus (ATCC25923)		*P. aeruginosa* (ATCC27853)	
	Exclusion	Displacement	Exclusion	Displacement
B. cenocepacia ET12	75.20 ± 29.44	46,63 ± 29,29	96.4 ± 0.29	93.48 ± 0.37

4.6. Identification of *B. cenocepacia* proteins on the supernatant expressed in LB and LB 0.4M NaCl cultures, from mid-log, late-log and stationary phase.

All proteins obtained in *B. cenocepacia* culture supernatants from 24h (late-log), 18h (mid-log) and 28h (stationary) phases were based on Uniprot database. Identification, accession numbers, subcellular locations and functions related are described on Table 5, 6 and 7, respectively. A total of 62 proteins were revealed on exoproteomes based on *Burkholderia* protein database, comprising 34 proteins associated to LB, 24 to LB 0.4M NaCl, and 04 common to both medias (Figure 12a).

Proteins identified are mainly related to the metabolism of the cell or to other intracellular processes (Figures 8, 9 and 10). Approximately 9% of total proteins were unknown or hypothetical. Four proteins were commonly found in LB and LB 0.4M NaCl, the first related to DNA recombination, transcription regulation, translation regulation (Q63TM8), the other two, to protein (Q1BRT3) or amino acid (B2JGX9) biosynthesis, and the fourth, to transcription regulation (Q13TY3) (Figure 12a). The first two mentioned here are also in the intersection of the stationary phase (Figure 11).

One protein associated to virulence was classified as “effector protein BipC” and is involved with type 3 secretion system (A3NLD). This protein was found in LB exoproteomes, but it was not detected in presence of LB 0.4M NaCl. It was found to be expressed at both mid-log (18h) and Stationary (28h) growth phases (Figure 12b). Exclusively related to LB, at the same growth phases, two other proteins were associated to amino acid biosynthesis (B4E634), and to cycle and cell division (A9AER8) (Figure 12b).

In comparisons between exoproteomes obtained at the same growth phase from culture media, we detected some proteins that were uniquely found in LB or LB 0.4M. For example, we found a protein related to the glycine betaine biosynthesis from choline and another related to degradation of capsule polysaccharide (Figure 11a) only in exoproteomes from mid-log phase (18h) obtained from LB. In the same culture media, during stationary phase (28h) proteins related to isoprene biosynthesis, protein folding and cell cycle / division were found only in LB (Figure 12a).

During mid-log phase (24h) we found proteins involved with translation, carbohydrate metabolism and biotin biosynthesis that were uniquely described in exoproteomes of LB (Figure 12). In presence of 0.4M NaCl (18h), we found exclusive evidences of cyanate metabolism, isoprene biosynthesis and nodulation during mid-log (Figure 11b), of proteins related to peptidoglycan biosynthesis during late-log, and others related to nitrogen metabolism and nickel insertion during stationary phase (Figure 12b).

Conserved processes, however, were detected in both culture media in proteins identified with different accession codes. During mid-log phase (18h), for example, the occurrence of activities related to amino acid, protein and nucleotide biosynthesis, including tRNA processing and protein transport, cell cycle and division and aromatic hydrocarbon catabolism (Figure 11) were described. At 28h, in both media, bacteria were found to be involved with DNA recombination, transcription- and translation- regulation, protein and with amino acid and lipid metabolism (including fatty acid and glycerol) (Figure 12).

Table 5 *B. cenocepacia* exoproteomes after 24h of growth (late-log phase).

Accession	Description	Subcellular Location	Function
LB_ late-log (24h)			
Q2T1Q3	ATP-dependent dethiobiotin synthetase BioD	Cytoplasm	Biotin Biosynthesis
A3MP60	30S ribosomal protein S9	Ribosome	Translation
B1YTD4	Anhydro-N-acetylmuramic acid kinase	Unknown	Carbohydrate metabolism
Q8GEK8	tive (5-formylfuran-3-yl)methyl phosphate synt	Unknown	Unknown
LB 0.4M NaCl_late-log (24h)			
B2T6U5	Bifunctional protein GlmU	Cytoplasm	Peptideoglycan Biosynthesis

Table 6 *B. cenocepacia* exoproteomes after 18h of growth (mid-log phase).
→ highlights virulence-associated proteins

Accession	Description	Subcellular Location	Function
LB_ mid-log (18h)			
Q13TR5	Sec-independent protein translocase protein TatA	Membrane (inner)	Protein Transport and Secretion
Q0BJB0	UPF0102 protein Bamb_0202	Unknown	Unknown
Q1BXC3	Phosphate import ATP-binding protein PstB	Membrane (inner)	Phosphate transport
B2JCN4	Phosphopantetheine adenylyltransferase	Cytoplasm	Coenzyme A Biosynthesis
A9AER8	Cell division topological specificity factor	Unknown	Cycle and cell division
Q9AI36	Phosphoheptose isomerase	Cytoplasm	Carbohydrate metabolism, biosynthesis and degradation of capsule polysaccharides
A9AGZ7	Histidine--tRNA ligase	Cytoplasm	Protein Biosynthesis
B4E634	ATP phosphoribosyltransferase	Cytoplasm	Amino Acid Biosynthesis
Q39D49	Endoribonuclease YbeY	Cytoplasm	Ribosome biogenesis, rRNA
A9ANA4	Exodeoxyribonuclease 7 small subunit	Cytoplasm	DNA catabolism
A9AN00	NAD/NADP-dependent betaine aldehyde dehydrogenase	Unknown	Glycine betaine biosynthesis from choline
B2JGX9	4-hydroxy-tetrahydrodipicolinate reductase	Cytoplasm	Amino Acid Biosynthesis
B2T605	tRNA (guanine-N(1)-)-methyltransferase	Cytoplasm	tRNA processing
Q3JVC0	UPF0234 protein BURPS1710b_1071	Unknown	Unknown
A3NLD4	→ Effector protein BipC	Secreted	Virulence
Q45696	2_4-dinitrotoluene dioxygenase system_ small oxygenase component	Unknown	Aromatic hydrocarbon catabolism
A4JH80	Adenylate kinase	Cytoplasm	AMP salvage
LB 0.4M NaCl_mid-log (18h)			
B4E852	Ribose-5-phosphate isomerase A	Unknown	Pentose phosphate bypass, non-oxidative branching
B2JIG2	Putative 4-hydroxy-4-methyl-2-oxoglutarate aldolase	Unknown	RNA metabolism regulator
Q13TY3	Transcriptional regulator MraZ	Nucleoid	Transcription Regulator
Q1BWS6	Ribosome-binding factor A	Cytoplasm	Ribosome Biogenesis
A4JBH8	4-hydroxy-tetrahydrodipicolinate reductase	Cytoplasm	Amino Acid Biosynthesis
Q1BRT3	Elongation factor Tu	Cytoplasm	Protein Biosynthesis
Q397R0	Acetaldehyde dehydrogenase 1	Unknown	Aromatic hydrocarbon catabolism
B1JTF1	30S ribosomal protein S18	Ribosome	Translation
B1Z5U9	Cyanate hydratase	Unknown	Cyanate Metabolism
P37335	Uncharacterized HTH-type transcriptional regulator BphR	Unknown	Transcription Regulator
Q39BA7	Ureidoglycolate lyase	Unknown	Nucleotide metabolism
A3P034	ATP phosphoribosyltransferase	Cytoplasm	Amino Acid Biosynthesis
Q145X1	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	Unknown	Isoprene Biosynthesis
A1V0B1	Ribose 1_5-bisphosphate phosphokinase PhnN	Unknown	Biosynthesis of 5-phosphoribose 1-diphosphate
Q2SVP3	Nod factor export ATP-binding protein I	Membrane (inner)	Nodulation
Q2SWQ8	Trigger factor	Cytoplasm	Cell cycle and division, protein folding, transport proteins
B2T2D1	Ketol-acid reductoisomerase (NADP(+))	Unknown	Amino Acid Biosynthesis
B4EAN4	tRNA (guanine-N(1)-)-methyltransferase	Cytoplasm	tRNA processing

Table 7 *B. cenocepacia* exoproteomes after 28h of growth (sta, stationary phase).
→ highlights virulence-associated proteins

Accession	Description	Subcellular Location	Function
LB_ sta (28h)			
Q1BRT3	Elongation factor Tu	Cytoplasm	Protein Biosynthesis
B2T3R7	50S ribosomal protein L31 type B	Ribosome	Translation
B2JG20	30S ribosomal protein S18	Ribosome	Translation
B2JGV1	30S ribosomal protein S9	Ribosome	Translation
A9AER8	Cell division topological specificity factor	Unknown	Cycle and cell division
B4E634	ATP phosphoribosyltransferase	Cytoplasm	Amino Acid Biosynthesis
A2S6B4	Peptidyl-tRNA hydrolase	Cytoplasm	Unknown
A9AJQ9	Trigger factor	Cytoplasm	Cell cycle and division, protein folding, transport proteins
Q39J12	60 kDa chaperonin 2	Cytoplasm	Protein folding
B2TCK0	HTH-type transcriptional regulator BetI	Unknown	Transcription
Q1BYL5	60 kDa chaperonin 1	Cytoplasm	Protein folding
Q9ZFE0	60 kDa chaperonin	Cytoplasm	Protein folding
Q13TY3	Transcriptional regulator MraZ	Nucleoid	Transcription
B2SXG6	diphosphocytidyl-2-C-methyl-D-erythritol kina	Unknown	Isoprene Biosynthesis
Q39JW3	UDP-3-O-acyl-N-acetylglucosamine deacetylase	Unknown	Lipid biosynthesis, lipid metabolism
B2JD95	Glycerol kinase	Unknown	Glycerol Metabolism
B2JHX4	Sec-independent protein translocase protein TatA	Membrane (inner)	Protein transport, Translocation, Transport
A3NLD4	→ Effector protein BipC	Secreted	Virulence
Q63TM8	Integration host factor subunit alpha	Unknown	DNA recombination; Transcription regulation; Translation regulation
Q146E7	Taurine import ATP-binding protein TauB 1	Membrane (inner and peripheral)	Transport
LB 0.4M NaCl_sta (28h)			
B2JGX9	4-hydroxy-tetrahydrodipicolinate reductase	Cytoplasm	Amino Acid Biosynthesis
B4EAZ1	50S ribosomal protein L31 type B	Ribosome	Translation
Q1BS24	ATP phosphoribosyltransferase	Cytoplasm	Amino Acid Biosynthesis
Q1BRT3	Elongation factor Tu	Cytoplasm	Protein Biosynthesis
Q39B48	Enoyl-[acyl-carrier-protein] reductase [NADH]	Unknown	Fatty acid biosynthesis
Q63TM8	Integration host factor subunit alpha	Unknown	DNA recombination; Transcription regulation; Translation regulation
A9AEY6	Peptidyl-tRNA hydrolase	Cytoplasm	Unknown
A9AHQ6	Putative 4-hydroxy-4-methyl-2-oxoglutarate aldolase	Unknown	RNA metabolism regulation
A4JEA9	UPF0178 protein Bcep1808_1605	Unknown	Unknown
A4JC39	Urease accessory protein UreG	Cytoplasm	Nitrogen metabolism, nickel insertion

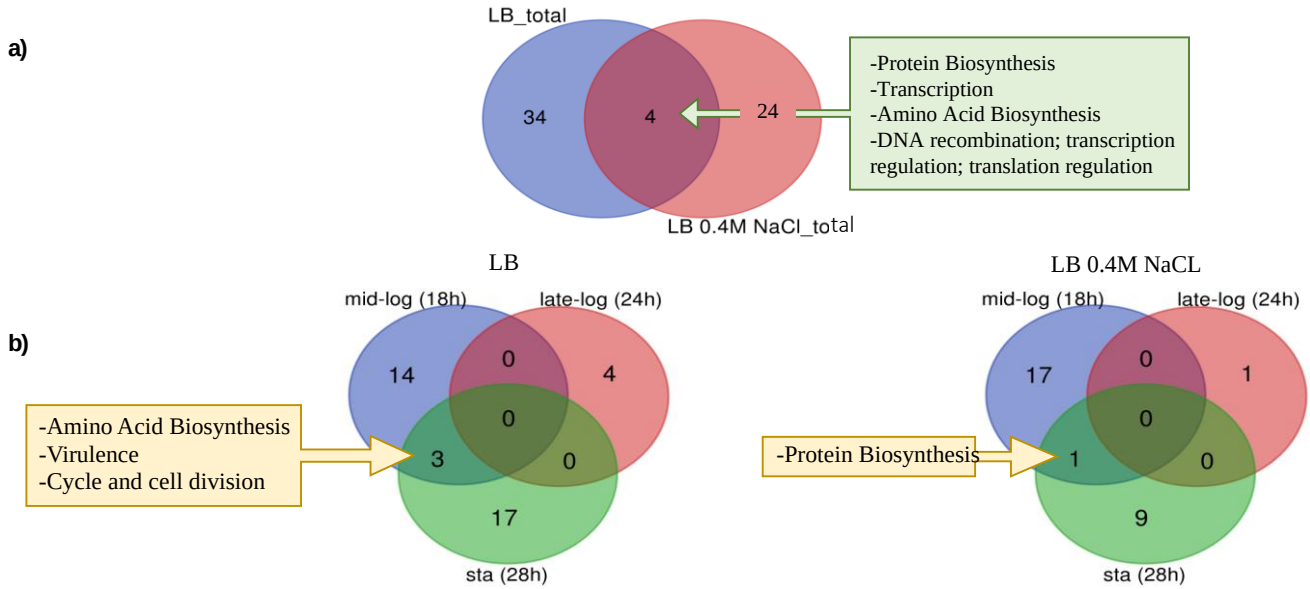


Figure 8 Venn Diagram representation of the total number of identified proteins recovered from supernatants of *B. cenocepacia* grown in LB and LB 0.4M NaCl.
(a) Proteins pooled according to the culture media. (b) Proteins pooled according to growth phase in mid-log (18h, blue circles), late-log (24h, red circles) and sta (28h, green circles). Green (a) and yellow (b) boxes indicate common protein functions in (a) media or in (b) growth phases indicated.

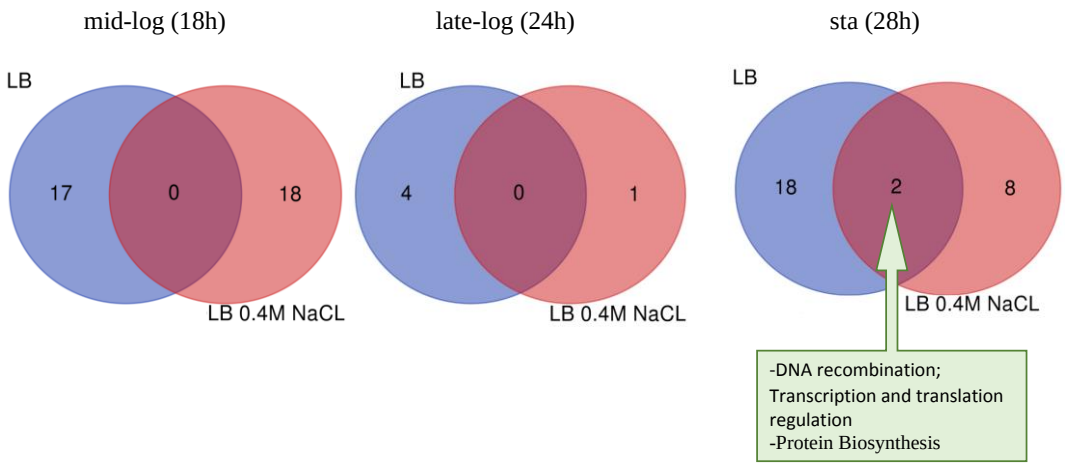


Figure 9 Venn Diagram representation of the total number of identified proteins recovered form supernatants of *B. cenocepacia* cultures at different growth phases. mid-log (18h), late- log (24h) and sta (28h).
Exoproteomes are indicated; blue and red circles represent exoproteomes of LB and LB 0.4M NaCl growth media, respectively. Green box indicates protein functions found in both culture media

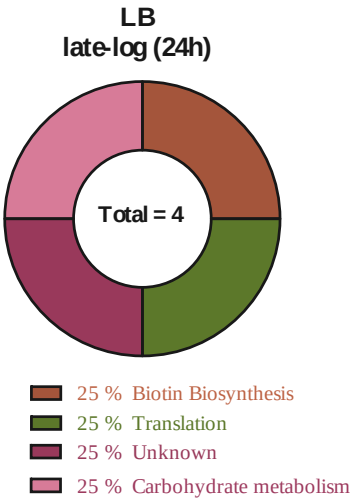


Figure 10 Overview of biological processes associated to *B. cenocepacia* exoproteomes identified based on Uniprot sequence, from late-log (24h) phase.
Percentages of biological process in each functional category are indicated in sections. Exoproteomes were obtained in LB. In LB 0.4M NaCl, only one protein involved with peptidoglycan biosynthesis was found (data not shown).

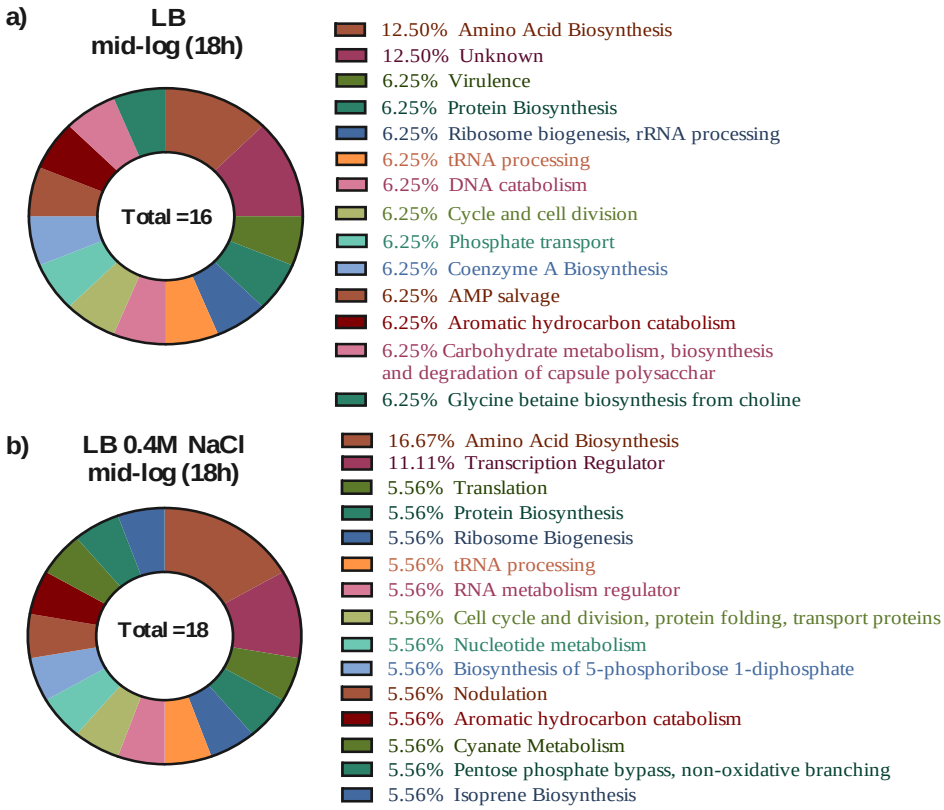


Figure 11 Overview of biological processes associated to *B. cenocepacia* exoproteomes identified based on Unipart sequence, from mid-log (18h) phase.
Percentages of biological process in each functional category are indicated in sections. Exoproteomes were obtained from (a) LB or (b) LB 0.4M NaCl culture media.

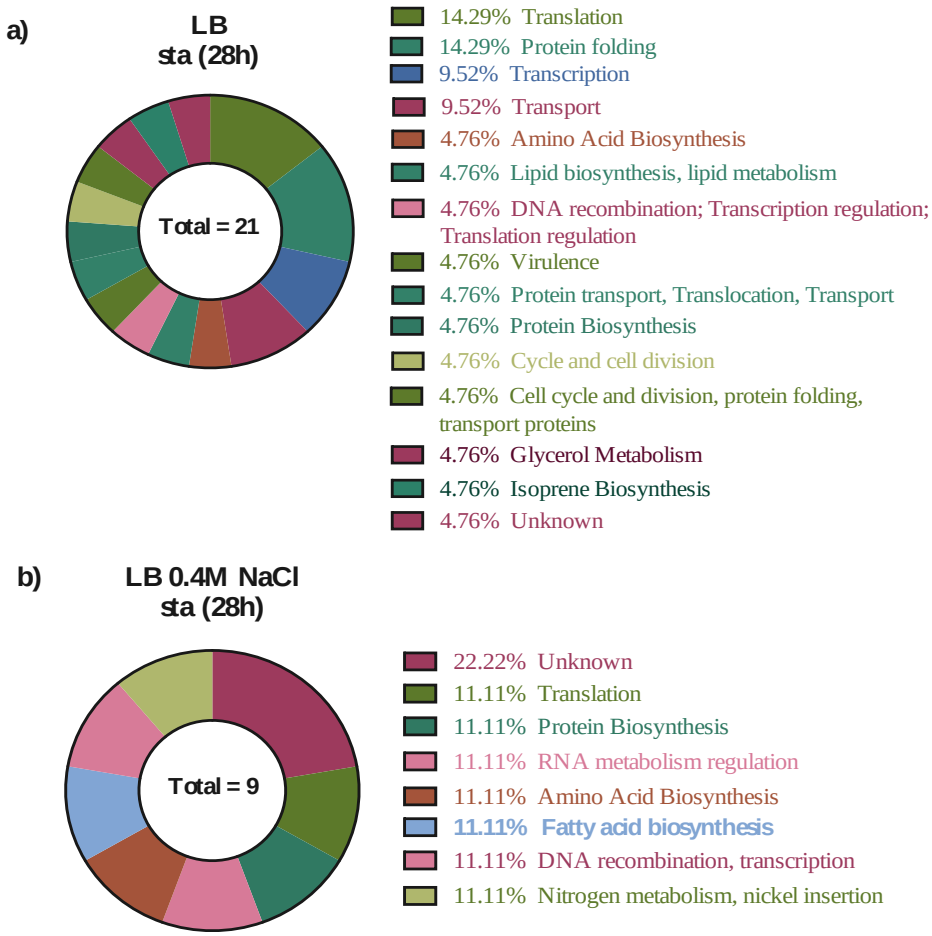


Figure 12 Overview of biological processes associated to *B. cenocepacia* exoproteomes identified based on Uniprot sequence, from Stationary (sta, 28h) phase.
Percentages of biological process in each functional category are indicated in sections. Exoproteomes were obtained from (a) LB and (b) LB 0.4M NaCl culture media.

5. CONCLUDING REMARKS

- *B. cenocepacia* J2315 takes 18h to reach mid-log phase; 24h and 28h to get at late-log and stationary phases, respectively.

- At higher concentrations of NaCl (0.3-0.5M) and NAC (2.0 g/L), *B. cenocepacia* growth is not inhibited, but it is slower in relation to LB used as a control.

- The biofilm was negatively affected in presence of 0.4M NaCl or 1g/L NAC. However, when these conditions were combined in LB, they increased biofilm during mid-log phase.

- More *B. cenocepacia* biofilm production was detected during stationary phase in comparison with mid-log phase.

- *B. cenocepacia* mucin adhesion was lower in presence of 0.4M NaCl, 1g/L NAC and 0.4M NaCl + 1g/L NAC during mid-log phase. At stationary phase, however, NAC combined with NaCl increased *B. cenocepacia* ability to adhere to mucin.

- *S. aureus* and *P. aeruginosa* showed stronger biofilms and more ability to adhere to mucin in comparison with *B. cenocepacia*.

- *B. cenocepacia* excluded and displaced *S. aureus* and *P. aeruginosa* strains at both growth phase (mid-log and stationary). During stationary phase, more than 90% of *P. aeruginosa* cells were displaced by *B. cenocepacia*.

- 62 proteins were found in exoproteomes of *B. cenocepacia* cultures, most of them related to metabolism and intracellular processes.

- The BipC effector protein related to virulence was only detected in exoproteomes of LB media. It was expressed at mid- log and stationary phase.

CHAPTER 2

In vivo analysis of *B. cenocepacia* virulence in zebrafish

1. ABSTRACT

Recent studies report that, after intravenous micro injection in zebrafish larvae 30 hour post fertilization, *B. cenocepacia* is phagocytosed by macrophages, in which they replicate, followed by bacterial dissemination and a robust pro-inflammatory infection that becomes rapidly fatal for the larvae (Mesureur *et al.*, 2017). It has not been analysed whether the presence of drugs, such as cysteamine, hypertonic saline concentrations, or conditions mimicking the CF lung composition affect *B. cenocepacia* virulence in the zebrafish infection model. ASMDM is a medium developed to mimic CF mucous, and hypertonic saline and cysteamine represent strategies available to treat CF patients. Therefore, we were interested in determining the effect of pre-culture of the epidemic strain *B. cenocepacia* K56-2 in LB containing 0.4M NaCl or ASMDM sputum medium prior to infection of the bacteria in zebrafish larvae, and analyse whether these pre-culture conditions may affect the production of bacterial factors involved in virulence in zebrafish. The effect of cysteamine, either injected (0.05mM and 0.1mM) or in the swimming water of zebrafish larvae (0.1mM, 1.0 mM), was also evaluated. Although NaCl at 0.4M was found to strongly affect physiological characteristics of *B. cenocepacia* itself, no significant effect on virulence was observed. qRT-PCR analysis of *il1b* and *cxcl8* genes showed that, when added to the swimming water of embryos, cysteamine (0.1mM, 1.0 mM) does not significantly affect anti-inflammatory responses in zebrafish. Also in ASMDM and cysteamine treatment, no significant differences in virulence was observed. In conclusion, virulence of K56-2 in zebrafish did not change as consequence of pre-culturing bacteria under higher osmolarity, in presence of ASMDM and of cysteamine, even treated or injected zebrafish. Our results contribute to a better understanding of *B. cenocepacia* mechanisms of infection in conditions similar to the CF lungs, using zebrafish as a model.

Keywords: virulence; *B. cenocepacia*; zebrafish; NaCl; cysteamine; ASMDM

2. OBJECTIVES

2.1. General

- To address aspects related to the virulence of *B. cenocepacia* using a zebrafish infection model.

2.2. Specifics

- To evaluate the effect of LB 0.4M NaCl, as follows:
    - To determine J2315 and k56-2growth kinetics
    - To estimate CFUs of k56-2 in LB and LB 0.4M NaCl under shaking
    - To investigate k56-2 viability (dead/live staining) and cell size

- To investigate the virulence in zebrafish model of bacteria cultivated in ASMDM (*B. cenocepacia* k56-2 and *B. stabilis*)

- To elucidate the effect of cysteamine on *B. cenocepacia* k56-2 infections of zebrafish, either by injecting or adding it to the swimming water
    - To test, as experimental control, the antimicrobial effect of cysteamine added at different concentrations (0.05, 0.1, 1.0 and 10 mM) for 1h, followed by 1% Triton-X-100 for 20 minutes

- To analyse if the addition of cysteamine to the swimming water triggers *il1ß* and *cxcl8* gene expression

3. MATERIAL AND METHODS

3.1. Bacteria strains and culture media

Bacterial strains and plasmids used in zebrafish infection assays are detailed in Table 8. The strains containing plasmid pIN29 (Vergunst *et al.*, 2010), encoding DSRed were used to visualize bacteria in real time. Bacterial stocks were kept in LB containing 15% glycerol at -80^{o}C.

Table 8 Bacterial strains and plasmids.

Bacterial Strains	Strain number	Descritption	Reference
Burkholderia cenocepacia K56-2	LMG18863	ET12, Toronto, Canada, CF[a]	*(Darling et al., 1998)*
Burkholderia cenocepacia J2315	LMG16656	ET12 index strain, Edinburgh, UK, CF	*(Govan et al., 1993)*
Burkholderia stabilis	LMG14294	Belgian CF patient, stable condition; detected in one other patient	*(Revets et al., 1996)*
Plasmids			
pIN29		DSRed reporter-containing plasmid, pBBR-derived; Cm^R	*(Vergunst et al., 2010)*

[a] CF, cystic fibrosis patient; Cm^R, chloramphenicol resistance

Lysogeny Broth (LB) was used according to Miller`s to cultivate bacteria. The amount of NaCl was modified in experiments to evaluate the effect of 0.4M NaCl on bacterial virulence. In this case, the modified media was used to determine CFUs counts of bacterial cultures, for live and dead cell viability assays, and for zebrafish assays (bacterial burden and survival).

In comparison with LB Vegitone (used for experiments described in chapter 1), originally, LB according to Miller's has a higher concentration NaCl (10g/L, instead of 5g/L). However, in experiments to evaluate the effect of NaCl, to be able to compare *in vitro* and *in vivo* results, we prepare LB Miller with 5g/L NaCl (equivalent to 0.086M, for control) and with 23.4g/L (LB 0.4M NaCl, our test condition). The other experiments (to test the effect of ASMDM and cysteamine in CFU counts obtained from bacterial cultures and in zebrafish assays) were carried out with the original concentrations of NaCl (10g/L). The original composition of LB according to Miller's was kept to compare with the standard already in use for zebrafish in the laboratory (Mesureur and Vergunst, 2014). Another important difference with the media employed in chapter 1 is that peptone was used here as carbon source in place of tryptone. The concentrations of peptone (in comparison to tryptone) and of yeast extract are the same (10g/L).

The artificial CF sputum media (ASMDM) was also used, and prepared according to Fung and colleagues (Fung *et al.*, 2010) with modifications described by Wijers (Wijers *et al.*, 2016), that are detailed below. Type II Mucin from porcine stomach (10 mg/mL; Sigma) and DNA from salmon sperm (1.4 mg/mL; Sigma) were dissolved in 100mL of sterile water, and then stirred and homogenized for 5 min at room temperature to dissolve.

After, bovine serum albumin (10 mg/mL; Sigma), potassium chloride (2.2 mg/mL; Fisher Scientific), sodium chloride (5mg/mL Fisher Scientific), diethylene triamine pentaacetic acid (5.9 µg/mL, Fluka Analytical), casein hydrolysate (5mg/mL; Merck), were dissolved in 70 mL of sterile water. Egg yolk emulsion (5mL/L; Sigma-Aldrich) was included.

Chloramphenicol (100 µg/mL) was added to prevent environmental bacterial contamination, as it is considered a broad-spectrum antimicrobial and the strains used in the experiments contain a plasmid with a cat gene. The pH was adjusted with 1M Tris-HCl (pH 8.5) to 6.5. Then, the volume was completed to 200mL. ASMDM was stored with antibiotics at 4 °C, for max 1 week. Before each use, ASMDM was warmed to 37 °C prior to addition of inoculum.

3.2. Culture and preparation of inoculum

Five mL of the respective media was inoculated with a loop of bacteria kept in glycerol stocks at -80º C and incubated overnight at 37º C on a rotary shaker. To grow wildtype strains carrying the pIN29 plasmid, media were supplemented with Cm 100 µg/L; for wildtype strains without the plasmid, Amp was added at 100 µg/L. Both antimicrobials were added to the media immediately before use. After growth, the optical density at 600nm was measured. Dilutions from OD 1 were prepared in PBS as a start point to obtain suspensions for the different experiments described below.

3.3. CFU count assay of cultures and growth curves

To obtain colony forming unit (CFU) counts for growth curves, bacteria were pre-cultivated in LB at 37ºC. From these pre-inocula, the suspensions were adjusted to OD 1.0 and aliquots were transferred to new culture media. However, for all other CFU counts assays, bacteria were directly transferred from cryotube stocks to the media used to grow the bacteria.

After growth, the suspension was adjusted to OD 1.0, serially diluted (10x), and plated on LB Agar plates (10uL, from 10^{-4} to 10^{-7}). Then, the number of CFU was obtained. Plates containing between 30-300 CFU were selected for counting. Time of growth and aeration are indicated hereafter, according to the experiment.

J2315 pIN29 growth curves were obtained in LB and LB 0.4M NaCl under shaking and static conditions. At 0h, 5h, 22h, 25h, 30h and 46h, aliquots from cultures were collected to construct the growth curves.

We have also obtained K56-2 pIN29 CFU counts, with shaking, in conditions that could potentially inhibit bacterial growth. At first, CFU's were determined in LB and LB 0.4M NaCl at 18h and 28h. Differences in CFU counts in medium containing NaCl were taken into account to prepare inocula for zebrafish injection experiments and normalized

to the LB control by dividing CFUs of bacteria grown in LB standard by the obtained CFU in LB 0.4M NaCl. This number was expressed as adjustment factor.

CFUs were also determined to estimate sensitivity to Triton-X-100 for the different strains. From the overnight growth obtained under shaking conditions, cells were prepared as described above and incubated for 20 minutes in 1% Triton-X-100 solution, followed by plating of serial dilutions on LB agar.

We have also determined K56-2 pIN29 resistance to 0.05, 0.1, 1.0 and 10mM of cysteamine. After overnight shaking cultures were obtained, and treated for 1 hour with cysteamine, followed (or not, in controls) by a second step in which the same cells were exposed for 20 minutes to Triton-X-100 1%, or as a control, with PBS, and CFU counts were estimated. All results obtained were corrected based on dilutions, and expressed as CFU/mL.

3.4. Live and Dead cell viability assay

The Live/Dead®BacLight™ Bacterial Viability Kit L7007 ThermoFisher Scientific was used to determine the viability of K56-2 WT in presence of LB or LB 0.4M NaCl. Bacterial cultures with 18h of growth were prepared in the same way as described above, under shaking. The instructions given by the manufacturer were followed with some minor modifications, detailed below.

A suspension equivalent to OD 1.0 was centrifuged at 3500 x g for 2 minutes, supernatant was removed, and the pellet was suspended in 1mL of saline solution (0.9 % of NaCl). From this suspension, a 20x dilution was prepared, and incubated for 1h at room temperature, mixing well every 15 minutes. The suspension was centrifuged again (3500 x g, 2min), and the pellet was suspended in a final volume of 100µL (saline).

A mixture Syto®9 and Propidium iodide (1:1) was mixed and added to the suspension (0.3µL to 100 µL). It was incubated in the dark at room temperature for 15 minutes, and 1µL was transferred to a clean slide and protected by a covering slip. Images were obtained with 100x oil immersion objective lens in an epifluorescence microscope.

Fluorescence was captured from both channels (green fluorescence for live, and red for dead bacteria), and microscope fields were randomly selected for bacteria cultivated

in LB and LB 0.4M NaCl. The numbers of live and dead cells were estimated. Microscopic fields with few bacteria were not considered for this analysis (n<5). The percentage of live and dead cells was then calculated. By merging pictures from both channels (green and red), a representative image was obtained using Adobe Photoshop. Only one experiment was done.

3.5. Cell size measurements

B. cenocepacia K562 pIN29 overnight cultures obtained in LB or LB 0.4M NaCl shaking at 37º C, were washed with saline solution (0.85%), fixed with paraformaldehyde (PF) 4% for 2 hours at room temperature, washed three times with PBS and observed under the microscope (100X objective oil lens, epifluorescence microscope). Representative images of each condition were obtained. ImageJ software was used to estimate length and width (µm).

Cells from each field were checked (n=10 cells). In rare cases in which discrepant morphologies were found (brighter or bigger in size), the cells were included at the same proportion as originally found in the field. Scale bars (9 pixels/µm) were based on a known distance of a Neubauer chamber measured from an image taken with the same microscope. Then, width and length sizes of *B. cenocepacia* K56-2 pIN29 were calculated with the scale tool. Two independent experiments were done.

3.6. Ethics statement

Zebrafish (*Danio rerio*) were kept and handled in compliance with the guidelines of the European Union for handling laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). Zebrafish studies performed at VBMI are approved by the Direction Departementale de la Protection des Populations (DDPP) du Gard (ID 30-189-4). Infection experiments in this

study were terminated before the larvae reached the free feeding stage (>5 dpf) and did not classify as animal experiments according to the 2010/63/EU Directive.

3.7. Zebrafish lines and fish care

Experiments were performed using transgenic embryos: *Tg (CMV:GFPmap1lc3b)*^{zf15} [5] (He *et al.*, 2009) which can be used for visualization of autophagosomal structures; *Tg(mpeg1:mCherryF)*^{ump2} (Bernut *et al.*, 2014; Nguyen-Chi *et al.*, 2014), that express mCherry in macrophages; and *Tg(mpx:egfp)*^{i114} (Renshaw *et al.*, 2006), in which neutrophils are labeled by GFP.

Adult fish were kept at 28^{o} C, alternating dark and light cycles (10h - 14h, respectively). Eggs were washed and kept in E3 medium incubated at 29 C. The eggs were checked twice a day in the first 24h and unfertilized or non-developing embryos were removed. Embryos were kept in Petri dishes containing E3 medium (Mesureur and Vergunst, 2014).

3.8. Microinjection, survival assay and bacterial burden

The detailed protocol describing assays related to microinjection of zebrafish to assess virulence were described by Mesureur and Vergunst (Mesureur and Vergunst, 2014). Briefly, the eggs obtained were washed and kept in petri dishes containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$) at 29^{o} C. Twenty eight to 30 hours post fertilization (hpf) the eggs were dechorionated manually with forceps 1 hour before injections.

Prior to micro injections, embryos were anesthetized in a solution of 0.4% tricaine methanesulfonat (MS222) placed on 1.5% agarose/E3 plates to immobilize the embryos. In the experiments analyzing the effect of salt, bacterial inoculum was adjusted based on

OD by a factor of 2.5x in LB 0.4M compared to LB to standardize the number of injected bacteria (see Figure 13c in the results section for differences in CFU counts standardized per OD for the two different media).

Each embryo was injected intravenously with approximately 1nL of a bacterial suspension, containing phenol red to follow the injection, adjusted to 50, 100 or 200 CFUs, as described previously (Mesureur and Vergunst, 2014). The exact injection dose of each embryo was obtained by CFU plating of 5 sacrificed embryos, as indicated in each section, at t=0 and t=24h.

3.9. Fluorescence microscopy

To obtain real time images, embryos were anesthetized with 0.2 mg/mL MS222 diluted in E3 and transferred to glass bottom dishes (MatTek Corp., Ashland, MA) for direct visualization. Samples of bacteria grown in different media were visualized using covered glass slides, using 40, 63 and 100 oil immersion objectives.

Images were obtained using a Coolsnap fx (Roper Scientifique) camera connected to a Leica DM IRB inverted microscope (bright-field, differential interference contrast - DIC), and fluorescence imaging, equipped with GFP and DsRed filter sets). Nikon AZ100 equipped for bright field and fluorescent imaging, coupled with Coolsnap HQ2 (Roper Scientific) supported by MetaVue software was used to obtain zebrafish and bacterial images. Those were further processed using ImageJ.

3.10. RNA extraction, purification, cDNA synthesis and quantitative real-time PCR (qRT-PCR) and Gene expression analysis

RNA extraction, cDNA synthesis and qPCR analysis were performed as described previously (Mesureur and Vergunst, 2014). Briefly, larvae were incubated in E3 medium with 0, 0.1mM or 1mM cysteamine. Embryos (n=20 for each condition) were transferred

to 500uL of Trizol at room temperature, homogenized using pipetting up and down with narrow tips followed by vigorous shaking on a vortex until complete homogenization. They were immediately transferred and kept at -80o C until analysis. Each sample was submitted to RNA extraction according to previous reports (Cui et al., 2011).

The purification of the RNA obtained was carried out using the RNeasy MinElute Cleanup kit (BioRad®), and after, quantified using NanoPhotomoter. The peptidylprolyl isomerase A-like (*ppial*) housekeeping gene and the *cxcl8* and *il1b* cytokine inflammatory genes were amplified by qRT-PCR from mRNA extracts with the {Foward (5`-3`) and Reverse (5`-3`) primes described in Table 9. The ΔΔCt method was used for analysis of the data, and the *ppial* was used as a reference gene.

Mean relative gene expression levels obtained in each qRT-PCR analysis were expressed by column bar graphs and normalized to the corresponding values obtained for E3-treated treated control embryos. Criteria used to classify a gene as up- or down-regulated was a difference of at least two folds (p-value < 0.5) between conditions.

Table 9 Primers used for qRT-PCR experiments.

Gene	F (5'3')	R (5'-3')	Accession #
cxcl8	TGTGTTATTGTTTTCCT GGCATTTC	GCGACAGCGTGGATCT ACAG	ENSDARG 00000100007
il1b	GAACAGAATGAAGCA CATCAAACC	ACGGCACTGAATCCAC CAC	ENSDARG 00000005419
ppial	ACACTGAAACACGGA GGCAAAG	CATCCACAACCTTCCC GAACAC	ENSDARG 00000042247

3.11. Statistical analysis

Statistical analysis of growth curves, cell viability assays and cell size measurements were done using R version 3.6.0. All graphics, and statistical analyses for survival assay and bacterial burden were obtained in Prism Version 6. As described in chapter 2, we estimated trend lines and the 95% confidence intervals to compare the growth curves using OLS. Statistical analyses of live and dead cell viability assays, and cell size measurements were done with Wilcoxon test for two independent samples.

For survival assays, Log rank (Kaplan-Meier) with Bonferroni corrections (when applied) was used. CFUs were obtained and numbers were expressed as log (CFU/ embryo). For bacterial burden, the significance between groups was determined using one-way ANOVA, with Sidak's Multiple Comparisons test. qRT-PCR data were log2-transformed, and significance of the data was analysed using unpaired T-test with equal SD (error bars, SEM). Significance is indicated as: ns, non-significant, *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

4. RESULTS

4.1. Pre-culture in media with high osmolarity does not affect virulence of *B. cenocepacia* in zebrafish

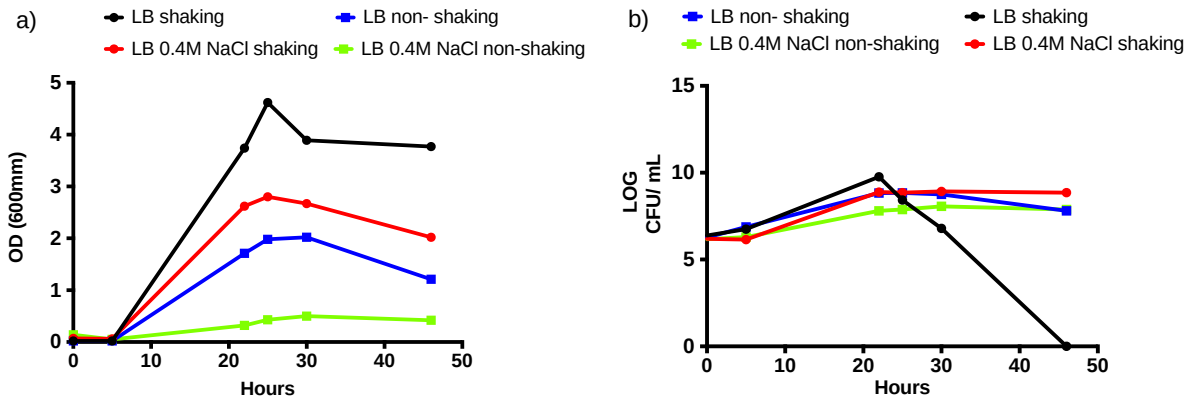
Previous *in vitro* work has demonstrated that variations in the concentration of salt induced the expression of virulence factors in *B. cenocepacia,* including cable pilli codified by a locus comprising 5 genes from which the *clb*A gene represents the major unit. Cable pili are known to mediate the adhesion of the mediating bacterial-host interactions (Tomich and Mohr, 2004).

High salt concentration also has been shown to increase biofilm formation on abiotic surfaces (Monteiro, 2012). To evaluate the effect of pre-culture medium with 0.4M NaCl on the virulence of *B. cenocepacia* using the zebrafish infection model, as described previously (Vergunst *et al.*, 2010), two highly virulent strains were employed, K56-2 and J2315.

Aeration, both with regular LB and LB complemented with 0.4M NaCl, resulted in a more rapid increase and maximum value for the absorbance at 600 nm (Figure 13a). The addition of salt to 0.4M clearly negatively affected the increase in OD, suggesting reduced bacterial growth. Plating of bacteria from the different conditions indicated that from 0 to 22h the log CFU/mL was slightly increased in LB (1.52), compared to 1.43 –fold increase at 0.4M NaCl under aeration.

Since the survival was measured using samples standardized to OD 1, this suggests that the bacterial growth is delayed at higher concentrations of salt (log 6.38 and 6.19 at T=0h compared to log 9.76 and 8.88 at 22 h for LB and LB 0.4M NaCl both shaking, respectively). This effect was measurable, and equally pronounced under static conditions (1.40 versus 1.26 fold change, respectively).

Unexpectedly, bacteria grown in regular LB with aeration seemed to have all died by 48 hours, while this was not the case for any of the other conditions. However, no statistical difference was detected based on time trends of growth curves and the 95% confidence intervals (data not shown), perhaps because we only considered one replica for each experiment. Although no statistical difference was detected between growth curves, Figure 13 shows a clear biological effect. In this case, the low number of observations probably affected the power of the statistical analysis.



c)

K56-2 pIN29			
Time	CFU/mL		Correction factor
	LB	LB 0.4M NaCl	
18h	1.2 x 10^9	4.9 x 10^8	2.5
28h	1.7 x 10^9	1.1 x 10^9	1.6

Figure 13 Growth efficiency of *B. cenocepacia* under high salt conditions.
In all conditions, bacteria were grown in a preculture and inocula were adjusted at T=0h to OD 1.0. From this, 0.1 mL was diluted to 4.6 mL. *B. cenocepacia* J2315 (pIN29) was grown in LB or LB 0.4M NaCl, both in shaking and non-shaking conditions (Data shown are representative of one experiment). (a) Growth curve expressed as OD 600nm and (b) the corresponding log10 (CFU/mL OD1) obtained (c) *B. cenocepacia* K56-2 (pIN29) was grown in LB with standard and high salt conditions for 18 and 28 hours on a rotary shaker. Based on the number of CFU obtained in the dilution the ratio between CFU in LB and LB 0.4M NaCl was determined (1 experiment). In order to inject the same number of live bacteria (CFU) for each treatment during zebrafish infections, the factor of adjustment was applied for future experiments. See material and methods for statistical analyses.

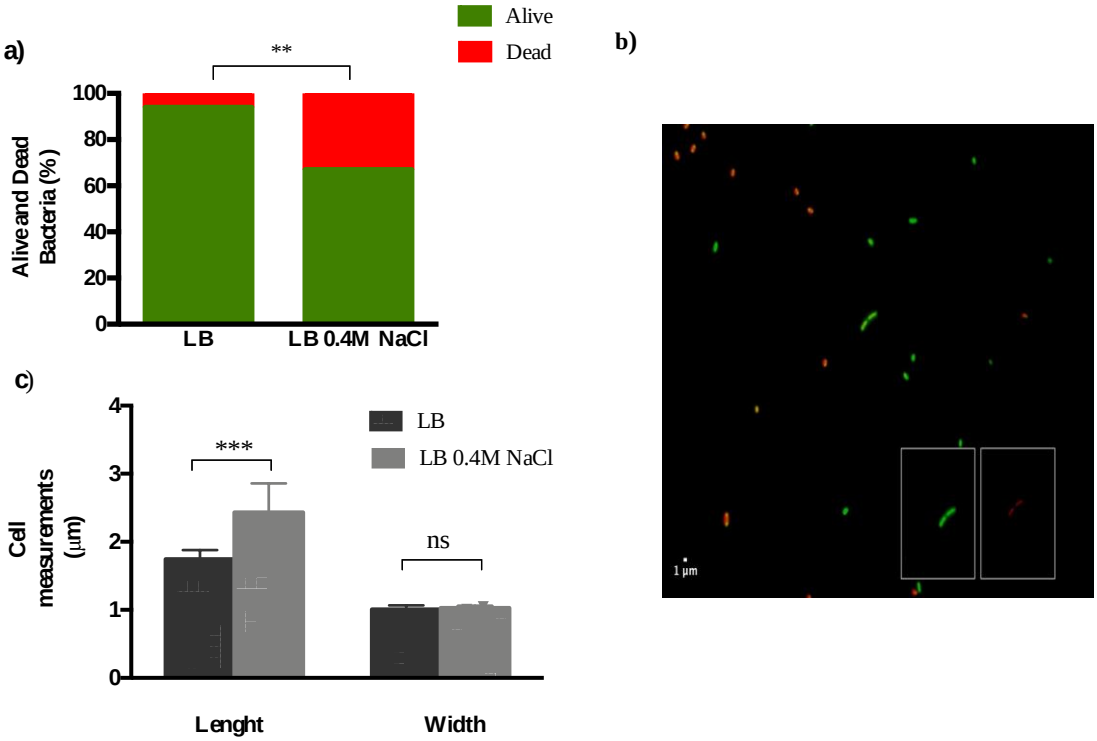


Figure **14**: Loss of viability and increase in size of *B. cenocepacia* K56-2.
Bacteria were cultivated in LB and LB 0.4M NaCL for 18h under shaking conditions. a) Viability of K56-2 WT was determined by Live/Dead Cell Viability Assays Thermo Fisher Scientific®. At least 9 fields of each condition were analysed. Microscopic fields containing less than 5 cells were not considered representative. Columns represent percentages of the averages of live (green) and dead (red) bacteria obtained in LB (n=10 fields) and on LB 0.4M NaCl (n=14 fields). This experiment was performed only once. b) To illustrate live and dead bacteria (as determined in a), the image shows an overlay of green and red images captured of J2315 after an overnight culture in LB 0.4M NaCl. The inset shows the individual red and green images for an individual bacterium. Scale bar, 1µm c) Length and widths of *B. cenocepacia* K56-2 pIN29. Cells were fixed with 4% paraformaldehyde. Six microscopic fields were analysed per condition, and from each image, measurements of 10 different cells randomly selected were taken. The graphs show measurements combined from two independent assays. Statistical analyses were done with Wilcoxon test for two independent samples. ** $p \leq 0.01$; *** $p \leq 0.001$; and ns: not significant.

Using dead/live staining kit (Figure 14a) we found that about 30% of the bacteria were dead when grown for 18 hours in LB 0.4M NaCl, compared to LB ($p \leq 0.01$). We also observed that cells cultivated in LB 0.4M NaCl (Figure 14c) were about 0.4µm higher in length comparison with measurements obtained for K56-2 pIN29 cultivated in LB ($p \leq 0.001$). However, no differences were detected in bacterial widths between LB and LB 0.4M NaCl (not significant).

Additionally, high osmolarity seemed to influence the arrangements of cells, as it was observed that cells cultivated in LB 0.4M NaCl appeared in small aggregates, instead of isolated as observed in cultures using LB as media (data not shown).

To better understand the influence of high osmolarity on the bacteria in the context of *in vivo* infections, K56-2 carrying plasmid pIN29 was cultivated in LB or LB 0.4M NaCl, and injected in zebrafish embryos with a calculated average of 50 CFU per embryo. Bacterial counts of individually treated embryos and survival assays were performed and the results are shown in Figure 15.

As can be seen in Figure 15a, the inoculum differed significantly between the treatments at T0h ($p \leq 0.05$). This makes it difficult to draw any firm conclusions based on the survival assay, which suggests that there is not a large difference in virulence between bacteria pre-grown in LB, or LB complemented to 0.4M NaCl (Figures 3 b).

As we use Triton-X-100-1% to disrupt embryos with a pestle, we have investigated whether this solution could affect survival of bacterial cultivated in LB 0.4 NaCl. No major differences were detected after treating overnight cultures of K56-2 pIN29 for 20 minutes with Triton-X-100-1% (Figure 16). CFUs obtained from cells after 20 minutes of treatment with Triton-X-100-1% were almost the same to non-treated cells.

We have also noticed that cells cultivated in LB 0.4M NaCl showed aggregation and were larger in size, which may result in injection of smaller volumes due to clogging of the injection needle. However, differences seen in cell sizes and growth rates in LB 0.4 NaCl makes it difficult to draw any direct comparisons in the virulence.

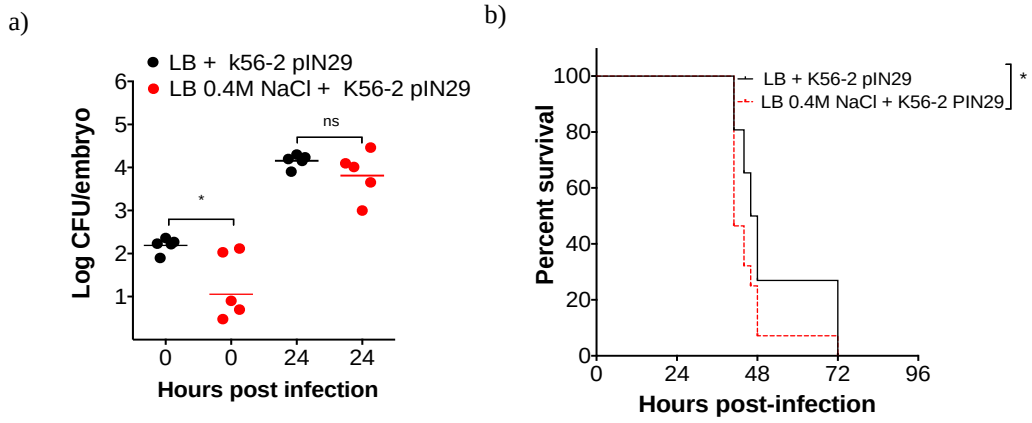


Figure 15 Virulence of *B. cenocepacia* K56-2 pIN29 after growth in LB 0.4M NaCl in zebrafish.

Embryos were injected in the blood circulation at 30 hours post fertilization with *B. cenocepacia* K56-2 pIN29 cultivated in LB or LB 0.4M NaCl with an inoculum of ~166 CFU (SD= 54,95) and ~51 CFU (SD=62.85), respectively (a, b). Bacterial burden (a) was determined for a total of 5 embryos for each condition and time point. Dots represent CFU per embryo. Geometric means are shown. (b) Embryo survival following infection. n=54 (b). Kaplan-Meier (Log rank test) was used as statistic method for survival, and one-way ANOVA with Sidak`s multiple comparisons test was used to determine significance between for bacterial burden. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ and ns: not significant. One experiment was performed.

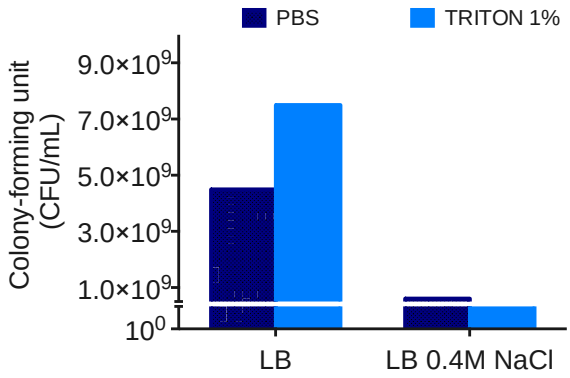


Figure 16 Osmotic stress does not increase sensitivity of *B. cenocepacia* k56-2 pIN29 to 1% Triton-X-100.
CFU counts were determined after an overnight culture under shaking conditions. Values (CFU/mL) obtained in one experiment are shown; the graphic is representative of two experiments performed.

4.2. Analysis of the effect of pre-culture in ASMDM (modified artificial sputum-media) on acute and persistent infection with Bcc strains

ASMDM is a medium developed to mimic conditions found in the lungs of patients with cystic fibrosis (Fung *et al.*, 2010). Several studies have demonstrated that virulent strains of *Burkholderia* sp. completely change their proteomic and transcriptomic profile when present in the airways of patients with cystic fibrosis between different stages in long-term infections (Moreira *et al.*, 2016).

Otherwise, the exact mechanism of virulence remains unclear (Jorge H. Leitão, 2017). For this reason, a medium that resembles conditions associated to airways of cystic fibrosis patients could be an interesting tool to advance virulence studies regarding interactions between bacteria and their host.

Using zebrafish, the effect of pre-cultivation in ASMDM on the virulence of K56-2 pIN29 and *B. stabilis* LMG12494 was evaluated. While K56-2 has been shown to cause acute fatal infection (Vergunst *et al.*, 2010), and Figure 17), *B. stabilis* causes persistent infection, characterized by bacterial survival and replication in macrophages in the absence of bacterial dissemination and pro-inflammatory signaling. Results of pre-growth in ASMDM are shown in Figure 17.

As shown in Figure 17 a and c, the amount of bacteria from the different treatments injected in embryos was highly similar (Figures 5 a and c). Survival assays showed that overnight growth of bacteria in AMSMD prior to infection does not affect acute virulence of *B. cenocepacia* K56-2, nor persistent infections caused by *B. stabilis* in zebrafish, as shown in (Figure 17 b, d). Results showing rapidly fatal infection for K56-2 and persistent, non-fatal infection for *B. stabilis*, are in agreement with previous studies (Vergunst *et al.*, 2010).

Since ASMDM medium may contain pro-inflammatory stimuli that may not be removed completely upon washing bacteria prior to injection in PBS, we analysed the effect of injection of small volumes of ASMDM with bacteria cultivated in LB. The result showed slightly increased embryo survival, however this control experiment was performed only once (Figure 17 b). As described above, the effect of Triton-X-100 1% was also evaluated after growth in ASMD (1 experiment), but no differences in bacterial sensitivity to Triton 1% were observed (data not shown).

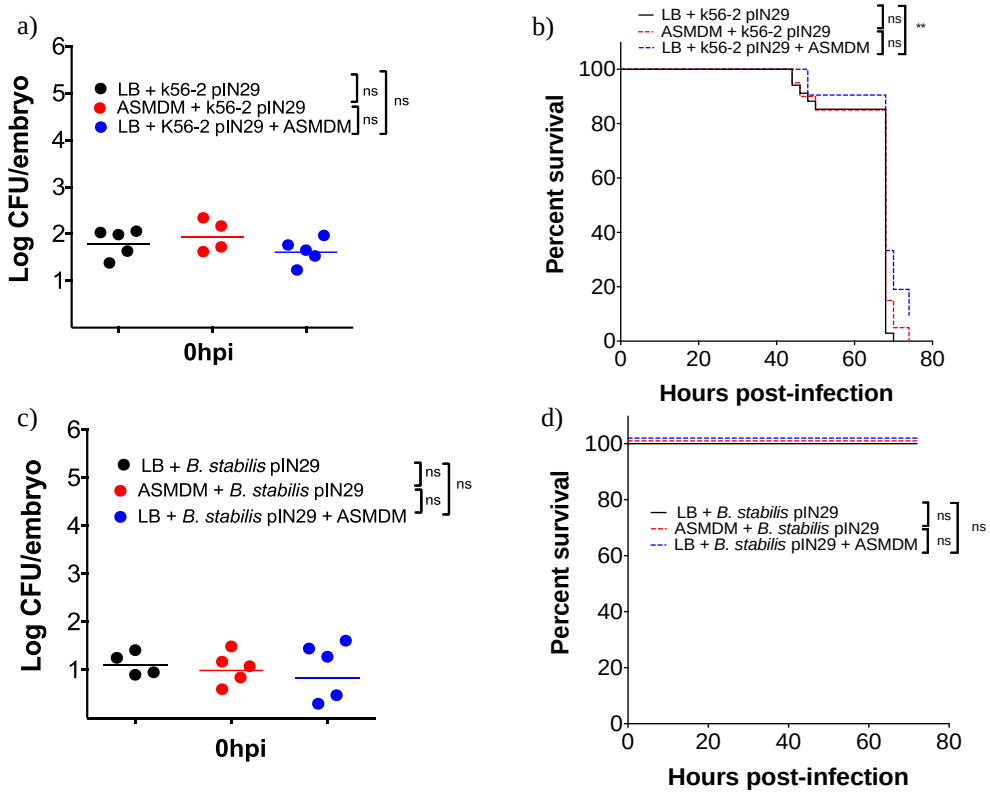


Figure 17 Effect of ASMDM on virulence of *B. cenocepacia* K56-2 pIN29 and *B.stabilis* pIN29.

Bacteria were grown in ASMDM, and in LB as control, diluted in PBS or AMSMD pellet. Embryos (Lc3-GFP) were injected with ~77 CFUs (SD=40,72), ~93 CFUs (SD=89,14) and ~49 CFUs (SD= 28,64) of *B. cenocepacia* k56-2 pIN29 (a,b), and with ~15 CFUs (SD=8,46), ~14 CFUs (SD= 10,52) and ~19 CFUs (SD= 16.65) of *B. stabilis* BIN2030 pIN29 (c,d), from LB, and ASMDM (diluted in PBS or ASMDM),

respectively. Graphics show CFUs for 5 individually treated embryos, and geometric means (a,c). (b, d) Embryo survival. n=75 (b) and n= 97 (d). For statistics, Kaplan-Meier (Log rank test) was used and corrected by the method of Bonferroni. ** $p \leq 0.01$, and ns: not significant. For bacterial burden, one-way ANOVA with Sidak`s multiple comparisons test was employed. One representative experiment from a total of three is shown for k56-2 pIN29 (a, b), as well results obtained for *B. stabilis* in one independent experiment performed (c, d).

4.3. Cysteamine does not change *B. cenocepacia* virulence neither pro-inflammatory cytokine expression in zebrafish larvae

The role of macrophages was described to be critical in the pathogenesis during *B cenocepacia* infections in zebrafish (Mesureur *et al.*, 2017). As cysteamine was recently described to interfere with autophagy (Jeitner *et al.*, 2018), we tested its effect during acute infection with *B. cenocepacia* in zebrafish larvae. We used two different approaches: first we added the cysteamine to the culture water, and secondly, we injected small doses of cysteamine simultaneously with the bacteria during intravenous injection. Since it has been described that cysteamine has direct antimicrobial properties against *B. cenocepacia* after 24h of growth (Shrestha *et al.*, 2017), we first analysed whether incubation of the bacteria in cysteamine for 1 h, the time between preparation of inoculum and microinjection in all embryos, would have antimicrobial effects.

We have chosen to work with the concentrations of 0.05, 0.1, 1.0 and 10mM cysteamine based on previous work published by Emonem and co-workers (Elmonem *et al.*, 2017), who had previously found an increase in autophagy rate in a model of cystinosis zebrafish mutants at 0.1mM of cysteamine, (Elmonem *et al.*, 2017).

At first, we have evaluated if cysteamine could exhibit antimicrobial properties in the conditions used in our study. Results showed that there was no statistical difference between CFU obtained for bacteria treated with cysteamine in relation to non-treated control (PBS). We have equally analysed whether an additional treatment with Triton-X-100 would have a negative effect on bacterial survival, but no difference was observed compared to the PBS control (Figure 18).

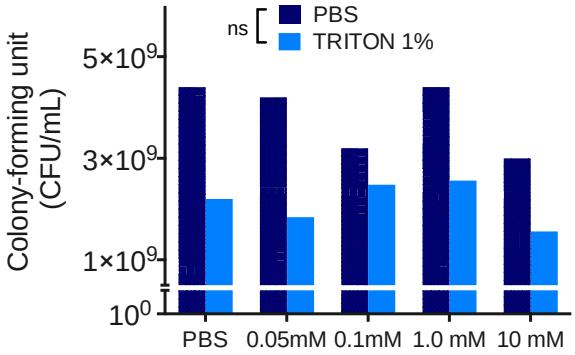


Figure 18 *B. cenocepacia* K56-2 pIN29 and its sensitivity to cysteamine.
Bacterial cells were grown overnight in LB and treated with different concentrations of Cysteamine in PBS (0.05, 0.1, 1.0 and 10mM) for 1h, and after that, for additional 20 minutes with Triton-X-100 1%. In both cases PBS was used as a control. Results obtained in one experiment performed.

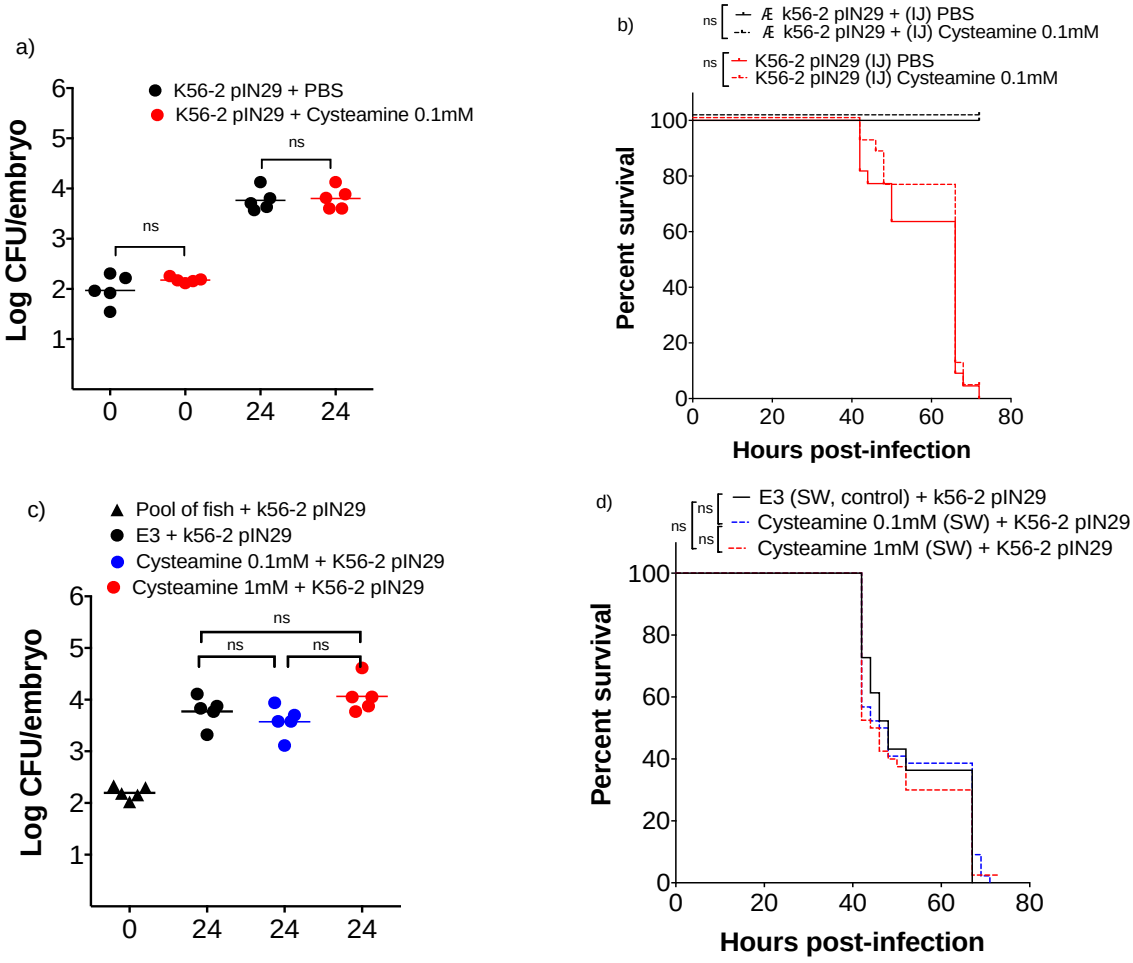


Figure 19 Virulence of *B. cenocepacia* k56-2 pIN29 in presence of Cysteamine.
(a,b) Zebrafish embryos (lc3-GFP, 30 hpf) were injected with ~116 CFUs (SD=67,45) and ~ 151 CFUs (SD=18.51) of *B. cenocepacia* K56-2 in PBS (n=22) or 0.1mM of cysteamine (n=25), respectively. As controls of survival assay, a set of non-infected embryos were injected only with PBS (n=19) or PBS + cysteamine (n=18). To check the number of injected bacteria, at 0hpi and 24hpi, CFUs obtained from 5 representative embryos per condition were counted (a). Bacterial load (a) and survival (b) were determined.

This experiment was performed once. (c, d) Zebrafish embryos (n=5) were injected with ~163 CFUs (SD=44,54) of *B. cenocepacia*. Embryos were pooled between times of injections to represent all conditions. The larvae were incubated in E3 fish water, complemented with cysteamine in the swimming water. Non-injected embryos with E3 (n = 22), 0.1 (n = 22) or 1.0mM (n = 21) of cysteamine in the swimming water did not die. Neither non-injected nor injected embryos in E3 (n=20), 0.1mM (n=20) or 1mM of cysteamine (n=18) did not show any anatomical or morphological changes (data not shown). A representative experiment of 3 is shown. Embryos scarified to obtain CFUs are represented with dots (a) or triangles (c), and geometric means are shown. For survival assays, Kaplan-Meier (Log rank test) was used, and corrections based on the method of Bonferroni were applied. For bacterial burden, one-way ANOVA with Sidak`s multiple comparisons test was used. ns: not significant. IJ: Injected; SW: Swimming water; Ø: controls not injected with k56-2 pIN29; SD: Standard Deviation; CFU: colony- forming unit; hpf: hours post fertilization; hpi: hours post infection. See material and methods for statistical analyses.

Thereafter, we tested if cysteamine interferes with the virulence of bacteria or with the immunological response of zebrafish. We used a solution containing cysteamine to resuspend bacteria and directly injected it in embryos. We also used solutions in the water used to bath the embryos, after they were injected with bacteria resuspended in PBS. Results obtained for survival assays and analysis of bacterial burden are shown in Figure 19. The results of one and two independent experiments of cysteamine either injected or added to the swimming water in different concentrations, respectively, show that cysteamine, did not significantly affect survival of zebrafish embryos.

4.4. Effect of cysteamine added to the swimming water on gene expression in zebrafish

To evaluate if addition of cysteamine (concentrations of 0.1 and 1mM) in the swimming water of 1-day old zebrafish larvae could trigger an immune response, gene expression of *il1b* and *cxcl8* was analysed by qRT-PCR. As can be seen in Figure 20, after 24h of incubation no significant differences in the expression of *cxcl8* or *il1b* were detected. These results are in agreement with the survival assays.

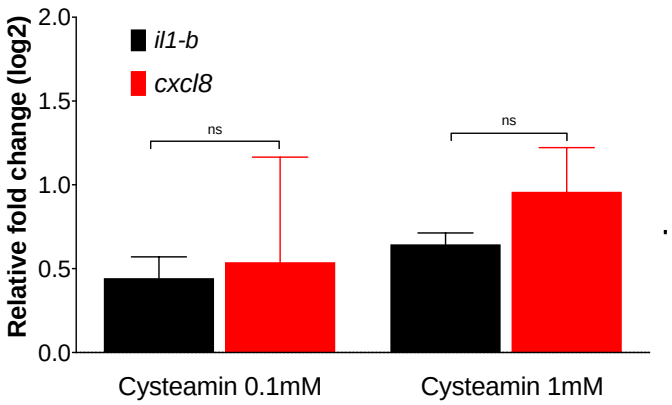


Figure 20 Pro-inflamatory cytokine gene expression does not change in zebrafish in response to treatment with cysteamine at 0.1 and 1.0mM in the swimming water.
qRT-PCR analysis of il1b (black bars) and cxcl8 (red bars) in embryos treated for 24h with 0.1 and 1.0mM cysteamine in the swimming water. Error bars represent a 95% confidence interval of the mean of two independent experiments. Two independent experiments are shown. Relative gene expression was analysed by 2- $\Delta\Delta\boldsymbol{Ct}$ method using PPIAL as a reference housekeeping gene to normalize data. Gene expression values of the different time points were calculated relative to a non-cysteamine, E3 bathed control. Difference in expression is indicated with respect to the E3 control group. Results are presented in a log2 fold change, and they were analysed using unpaired T-test with equal SD (error bars, SEM). Two independent experiments were done. ns: not significant.

4.5. Real-time observations of infection progress in zebrafish larvae

Real time analysis at different time points were performed, to follow the progression of the disease. Figure 21 illustrates representative images of embryos at 24hpi, injected with K56-2 pIN29 and treated with different concentrations of cysteamine in the swimming water. We observed that intravenous injected bacteria spread from the injection site (caudal vein, posterior blood island), reaching sites, as eyes and yolk, showed by presence of red fluorescence associated to pIN29 plasmid.

No obvious difference was seen between the different treatments. Due to the finding that both injection and bathing of embryos in cysteamine had no effect on bacterial load, embryo survival, or immune response gene expression, we have not further analysed possible differences in the behavior of host immune cells.

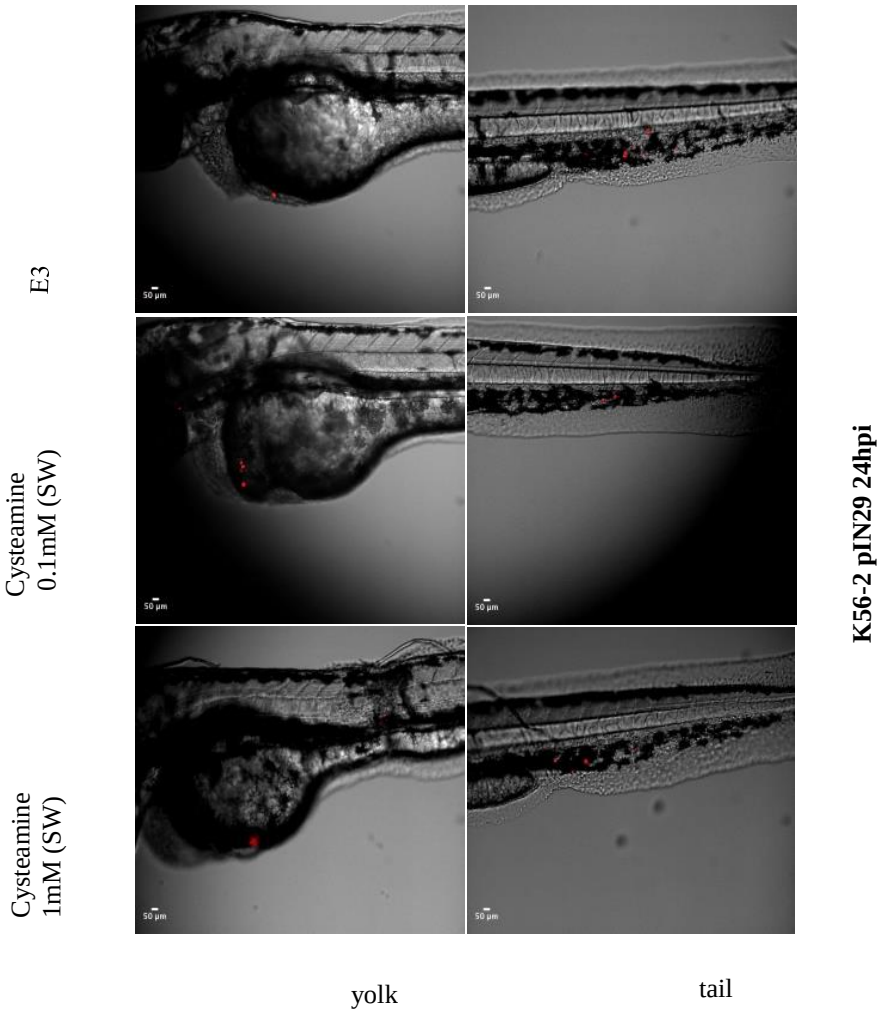


Figure 21 Real-time images taken at 24hpi showing bacteria in the yolk and in the tail of Tg(mpx-GFP) embryos injected with fluorescently marked *B. cenocepacia* k56-2 pIN29 (in red).
Injected embryos were either kept in E3 fish water, or E3 supplemented with cysteamine (0.1 and 1 mM). Real-time images captured with a camera attached to an epifluorescence microscopic,. Scale bar, 50µm.

5. CONCLUDING REMARKS

- J2315 showed less growth at higher concentrations of salt (0.4M NaCl), and this effect was enhanced under static growth conditions. This effect was reproducible for K56-2 pIN29.

- Under conditions of high osmolarity, *B. cenocepacia* K56-2 is less viable, and has an increased length.

- Despite adjustments applied to correct the amount of live bacteria injected in embryos, differences detected in growth curves and on cell sizes for bacteria cultivated in presence of LB 0.4M NaCl made it difficult to compare the effect of high osmolarity in virulence using zebrafish as an infection model.

- The sensitivity of the bacteria to 20 min treatment with Triton 1% was not different by cultivation in LB 0.4M NaCl compared to LB.

- Pre-culture in ASMDM sputum medium has no effect neither in the virulence of acute strains, represented by *B. cenocepacia* K56-2 pIN29, nor for persistent strains, as *B. stabilis* in the embryo infection model.

- Cysteamine does not have any measurable effect in zebrafish infections in the conditions employed in the study, as demonstrated by survival assays, by analyses of gene expression of *il1b* and *cxcl8* (qRT-PCR), and real time studies.

DISCUSSION

1. Chapter 1: *In vitro* assays

During stationary phase more biofilm production and mucin adhesion were detected in comparison with mid-log phase. In agreement to this, Saa and colleagues observed when they assessed *B. cenocepacia* J2315 by transcriptomic profiling, that upregulation of extracellular virulence factor genes are more pronounced during stationary phase (Sass *et al.*, 2013). Additionally, more than 25% of the global transcripts was found to change in response to the stationary phase and under limited oxygen atmosphere or growth arrest as well (Sass *et al.*, 2013).

These results suggest that bacteria does not start its virulence factor production immediately after starting to grow. It could indicate a similar effect on the pulmonary environment of the host which could indicate that *B. cenocepacia* become more virulent after a first step of adaptation to the environment of the host.

Non-shaking cultures were used to grew bacteria to reduce the levels of oxygen, although traditionally *B. cenocepacia* is known as non-fermenting obligatory aerobic microorganism (Vandamme *et al.*, 2003). We decided to use this condition to get closer to the CF lungs environment. The sputum interface is characterized by an oxygen gradient (Yang *et al.*, 2011) that can show very low rates especially within the mucus, that can reach 0% of oxygen (Yang *et al.*, 2011).

A high occurrence of strict anaerobic bacteria is reported in association to the CF sputum, which is in agreement with an environment with reduced oxygen level. In some cases, the amount of strict anaerobic bacteria can be equivalent to the number of aerobic pathogens related to CF pulmonary infections (Tunney *et al.*, 2008).

The growth curves obtained here endorsed the ability of *B. cenocepacia* to growth, even under non-shaking conditions. J2315 started the logarithmic phase only after 6h it was inoculated, entering in the decline phase early, with only 35h. This is in harmony with the recent description of a novel low-oxygen-activated locus that enable *B. cenocepacia* survive in the absence of oxygen , in conditions similar to the CF lungs (Sass *et al.*, 2013).

The concentrations of NAC and NaCl we used to cultivate bacteria were chosen based on reports found in the literature. In a recent clinical trial, authors have defined a treatment using oral NAC at a dose of 120 mg twice per day (Skov *et al.*, 2015), showing an increase in lung function. Also, the concentrations practiced for *in vitro* studies with gram-negative bacteria usually employ NAC varying from 0.5 to 2.0 g/L (Blasi *et al.*, 2016).

Salt stress is also a condition naturally found to occur in CF patients, as 40%-50% more NaCl are detected in CF sputum or CF ASL samples in comparison with healthy individuals, which show 7.4g/L of NaCl (Joris *et al*., 1993; Grandjean Lapierre et al., 2017). Besides, the use of HS 3-7% to nebulize patients during treatment can increase much more the NaCl concentration. We cultivate bacteria at 0.4M NaCl (final concentration of 2.4%), which seemed to be a reasonable amount to start investigations as, to our knowledge, the bioavailability of NaCl after CF patients are nebulized with HS have not been reported yet. Thought, the NaCl concentration in patients under treatment with HS remains to be elucidated.

We seek for sub-inhibitory NaCl and NAC concentrations, as close as possible to the concentrations these substances reach in CF lungs. Growth curves obtained in LB media complemented with NaCl at 0.3M, 0.4M or 0.5M NaCl, and with NAC at 2g/L as well, showed a reduced growth rate and seemed to be a disadvantage for *B. cenocepacia*. Based on this, we settled the sub-inhibitory concentrations of these compounds and decided to select 0.4M NaCl and 1g/L NAC as sub-inhibitory concentrations to test *B. cenocepacia* virulence factor production without compromising too much the bacterial growth.

For the first time, we tested the effect of 1g/L NAC in a context with higher NaCl (0.4M). Both conditions were combined in the culture media to examine whether high NaCl levels expected to occur in CF lungs in association with NAC, could benefit CF patients by reducing *B. cenocepacia* virulence factor production.

Either biofilm production and mucin adhesion demonstrated to be higher in presence of NaCl and NAC. This is the pioneer study that shows that *B. cenocepacia* under salt stress and in presence of NAC increase virulence factor production. Preliminary results obtained here indicate that this effect must be further investigated deeply, as it could represent a big disadvantage for those thinking in the use of NAC as alternative treatment for CF patients.

At 0.4M NaCl, we observed that *B. cenocepacia* reduced both biofilm production and mucin adhesion. An opposite effect was observed by Tomich and Moher (2004) in a study developed with *B. cenocepacia*. Authors have suggested that high osmolarity (0.1M and 0.2M NaCl) have induced the adherence to the lower respiratory tract mediated by cable pilus (*cbl*) (Tomich and Mohr, 2004).

It had been also showed for *B. pseudomallei*, a closely related specie to *B. cenocepacia*, that neither flagella production nor biofilms are influenced in a NaCl range from 0.15 to 0.3M NaCl (Pumirat et al., 2017). However, in presence of NaCl, bacteria experienced an adaptative change, enhancing plaque formation, heat and oxidative resistance (Pumirat et al., 2017). Another study had showed similar results, with no effect of hypertonic saline 7% in *B. cenocepacia* mature biofilms (Narayanaswamy et al., 2019).

Results obtained in LB 0.4M NaCl fit in a theory proposed by Williams and colleagues for *P. aeruginosa*, which suggests this bacteria vary its phenotype in osmolarity stress, as presence of HS. *P. aeruginosa* would change from mucoid to a non-mucoid phenotype associated to brand clinical symptoms, and then, the non-mucoid, more virulent and osmotically sensitive colonies would take place the niche previously occupied by the mucoid phenotype (Williams et al., 2010).

For many pathogens, adhesins are mandatory for the pathogen during first stages of infection. They represent a special virulence factor that enable pathogens adhere to many sites, as intestinal and respiratory mucosa. Adhesion is indicative of higher virulence as well, as more mucin binding rates are associated to strains isolated from severe patients in which patient`s condition evolves to death (Sajjan et al., 1992).

It could be argued that we used mucin from porcine stomach, and not from respiratory system to test mucin adhesion and competitive assays. It is worth to highlighting that it is both mucins are considered equivalents. For instance, mucin from porcine stomach was also used by others in a media developed to cultivate bacteria in conditions mimicking cystic fibrosis lung sputum (Sriramulu et al., 2005). Another study showed evidences that CF (respiratory) and non-CF (intestinal) mucins are equivalents, as strains associated to the syndrome cepacia showed the same adhesion capability in both sources (Sajjan et al., 1992).

In our study, we showed in first-hand that even at low doses (1g/L) NAC reduced *B. cenocepacia* biofilm production and mucin adhesion. There is no previous report exploring the effect of this compound against *B. cenocepacia* mucin adherence.

Activity against *B. cenocepacia* biofilm, on the other hand, had been explored in only one published paper, and its results obtained at 8g/L NAC were in agreement with to what was found here (Pollini et al., 2018). Pollini and colleagues have found that NAC inhibit two days-old biofilms and diminish *B. cenocepacia* growh (Pollini et al., 2018). The antimicrobial effect of this substance against *P. aeruginosa* (Zhao and Liu, 2010) and other pathogens (Olofsson et al., 2003; Moon et al., 2015; Abdel-Baky et al., 2017) have also been reported. Moreover, a systematic review highlights its effect on inhibition, destruction (early and mature stages) and in reducing the viability of biofilms formed in respiratory tract infections (Blasi *et al.*, 2016), and indicates its success as an anti-biofilm strategy (Dinicola *et al.*, 2014).

Polymicrobial infections are described to occur in CF and represent a good place for interactions among microorganisms (Layeghifard *et al.*, 2019). For instance, it was supposed that *S. aureus* exerts an effect against *P. aeruginosa* that could prevent the reinfection by this latter and would result in an increase in the life expectancy of CF patients of about 5 years of (Peters *et al.*, 2012).

By the competitive assay, we found that *B. cenocepacia* exert a negative effect on both *S. aureus* or *P. aeruginosa*. In presence of *B. cenocepacia* these pathogens were found to be impaired to assess the mucin adhesion sites, as revealed by the exclusion assay. Moreover, even when *B. cenocepacia* was put in the system after these pathogens were already occupying mucin sites, which at first could be considered a disadvantage, *B. cenocepacia* has shown ability to efficiently displace both *S. aureus* and *P. aeruginosa*. *B. cenocepacia* was found to disrupt *S. aureus* biofilms when they are co-cultivated (Thompson, 2017), which represents another evidence of the negative effect exerted by *B. cenocepacia* that is in congruence with our findings.

We suppose that the observed inhibition exerted by *B. cenocepacia* on *S. aureus* and *P. aeruginosa* might have a clinical relevance. As explained before, microorganisms associated to CF infections are related to the individual's age, whereas *S. aureus* appears at first ages and is followed by *P. aeruginosa,* and *B. cenocepacia* during adulthood (Harrison, 2007). Whether we consider that *B. cenocepacia* appear at latter ages in CF patients, and that infections caused by this microorganism are usually fatal, it makes sense to consider that *B. cenocepacia* exerts a deleterious effect on other pathogens when they compete for the same niche in the host.

B. cenocepacia ability to displace and exclude *S. aureus* and *P. aeruginosa* could also trigger effects on the other microorganisms. For example, to compensate the

difficulty to gain access to mucin, *S. aureus* and *P. aeruginosa* would react against it by the use of different strategies.

Supporting our theory, based on a model of CF pulmonary disease, Bragonzi and colleagues have shown that *P. aeruginosa* biofilms increase biomass and change the architecture during co-infection with *B. cenocepacia* (Bragonzi *et al.*, 2012), which might represent a good strategy of *P. aeruginosa* to persist and resist *in vivo*. *Burkdoleria cepacia*, that is close related to *B. cenocepacia*, has been shown to sense signs produced by *P. aeruginosa* (Riedel *et al*., 2001).

An unidirectional cross-talk system mediated by N-acylhomoserine-lactone molecules known to regulate biofilms was described during mixed biofilms between *B. cepacia* and *P. aeruginosa* (Riedel *et al.*, 2001). As *B. cenocepacia* and *B. cepacia* are phylogenetically related, it could also exist a similar system in *B. cenocepacia*.

By proteomics, we identified BipC, a protein involved with virulence. It was found here that it is expressed in both phases in *B. cenocepacia* cultures grown in LB. This protein was found not to be expressed in presence of NaCL at 0.4, which is in accordance with preliminary results obtained in mucin adhesion and biofilm production assays that indicated bacteria cultivated in 0.4M NaCl reduce virulence factor production.

This protein is described previously to be associated with secretion system type III and mutants for this system in *B. cenocepacia* J2315 have shown to be attenuated in virulence in murine (Tomich *et al.*, 2003), which suggests its role in host pathogenicity. Contrary to our findings, by total transcriptomic associated to western blot analysis BipD, that belongs to the same locus as BipC, was detected in cultures of *B. pseudomallei* cultivated under salt stress (0.3M NaCl), but not in salt-free media (Chin *et al.*, 2010). They also found that the invasion efficiency was higher in presence of salt (Chin *et al.*, 2010), which indicate that species belonging to the same genera could show opposite response under salt stress.

We considered that, moreover, few proteins were recovered from supernatants of *B. cenocepacia* cultures. Besides, the vast majority of the proteins identified were classified as intracellular, although we have tried to obtain only secreted cellular fractions. Cellular fractions of *B. cenocepacia* J2315 proteins were characterized elsewhere in *B. cenocepacia* clones retrieved from later stages of CF lung infections (Madeira *et al.*, 2013).

In general, their functions were similar to the ones described in our results, and were related to metabolism, iron homeostasis, nucleotide synthesis, translation and

protein biosynthesis and folding, and envelope biogenesis, for instance (Madeira *et al.*, 2013). This could be explained by the fact that at 18h, 24h and 28h, many cells were lysed, and that supernatant obtained from cultures were contaminated with cells. Results obtained indicate the need to adapt the protocol to avoid intracellular proteins in supernatants. After centrigation, supernatants could be filtrated with 0.22 µm membranes to improve the detection of proteins associated to the supernatants which are fewer and could be underdeteced in presence of high amounts of intracellular proteins. This experiment must be repeated, but due to technical problems, it was not possible to repeat it on time in order to confirm results.

NAC and Hypertonic saline have emerged as two simple, safe, and cost-effectiveness alternative that become to be investigated for CF and had shown many clinical benefits (Tirouvanziam *et al.*, 2006; Conrad *et al.*, 2015). Hipertonic saline, on other hand, has shown to improve the mucociliary clearance and the lung function (Donaldson *et al.*, 2006). They have been showing many benefits in clinical trials in the last years. The presence of a thick mucus is one of the hallmarks of CF clinics associated to pulmonary exacerbations, and both conditions, individually, seemed to be promising to act in alleviating symptoms, as they are been already used as mucolytic agents.

Our data endorsed effectiveness associated to the use of NAC and HS, as when these conditions were individually added to the media, they reduced *B. cenocepacia* virulence factor production. Inversely, data obtained with NAC associated to NaCl alarm for a possible negative effect on host, which should discourage those who must think in adopt NAC as an alternative treatment in patients with higher NaCl concentrations, as in CF.

Biofilm and mucin adhesion in conditions mimicking sub-inhibitory doses of NAC in CF lungs under treatment showed to increase virulence factors *in vitro*. However, before drawing a definitive conclusion about the effectiveness associated to the use of NAC in CF lungs, these results must be confirmed and expanded. Still, interactions between *B. cenocepacia* and other pathogens had showed that when *B. cenocepacia* is present, these bacteria get advantage over *S. aureus* and *P. aeruginosa*, inhibiting their adhesion to mucin.

Finally, although virulence cannot be inferred based on *in vitro* tests solely, we can still use them as good tool to investigate conditions related to CF lungs. They can be employed on a simple, fast and powerful way to screen potential new strategies of treatments that could affect pathogen virulence. Preliminary data obtained in this chapter

has encouraged us to go further in investigations to test similar conditions in *in vivo* models, as these models are indispensable to infer about a pathogen virulence.

2. Chapter 2: *In vivo* assays

One of the currently most used concepts to define virulence is "the relative capacity of a microorganism to cause damage in a host" (Casadevall and Pirofski, 2003). Many animal models have been developed and used over the years, including mammalian models, as they are closer to humans than non-vertebrate models such as *C. elegans* and fruit flies. The zebrafish has been recognized as a potential model in research for more than 40 years (Vascotto *et al*., 1997). Especially in the last two decades, the small size and optical transparency of the embryos, as well as the close homology to the human immune system have boasted its use to study the interaction of the immune system with microbes, greatly advancing our knowledge on host-pathogen interactions (Torraca and Mostowy, 2018).

These animals have also shown their usefulness as a "lower" vertebrate model to study infections caused by the Bcc complex, and helped to better understand differences between acute and persistent infections by different Bcc strains *(Vergunst et al., 2010; Mesureur et al., 2017; Gomes et al., 2018).*

We have shown in the previous section, that biofilm production *in vitro* by *B. cenocepacia* was reduced in the presence of salt stress. As shown for *P. aeruginosa*, it has been generally assumed that *B. cenocepacia* also forms biofilms in the lungs of CF patients, as it is a very good biofilm producer *in vitro*.

However, the presence and relevance of biofilms during infections in CF patients has not been clearly demonstrated for *B. cenocepacia,* in contrast to *P. aeruginosa* (Høiby *et al.*, 2010). Using cell culture assays, it has been shown for over 20 years that Bcc are intracellular bacteria, able to survive, and/or replicate in different types non-professional and professional phagocytic cells (mammalian, amoebes), and that they interfere with essential steps of phagolysosome maturation, cellular trafficking and autophagy (Abdulrahman *et al*., 2011; Al-Khodor *et al*., 2014; Valvano, 2015).

It has been suggested that the intracellular life stages of *B. cenocepacia* form a major contribution to the infection mechanism of these bacteria. In clinical studies, Bcc were also shown to be present in immune cells (Sajjan *et al.*, 2008; Schwab *et al.*, 2014). In fact, the most recent study described that *B. cenocepacia* was found in "micro colonies or as single cells within phagocytes or in mucus, and not in biofilm-like structures" in the lungs of heavily infected patients, and that co-infection of *B. cenocepacia* and *P. aeruginosa* resulted in the disappearance of *P. aeruginosa* biofilms (Schwab *et al.*, 2014).

Although zebrafish do not have lungs, and are therefore not a good model to study mechanisms mimicking the first steps of colonization and invasion of Burkholderia in humans, the major advantages of real time non-invasive analysis have shown that macrophages are critical for intracellular replication of Bcc bacteria *in vivo* and the development of a pro-inflammatory fatal response, in agreement with the clinical studies that identified the bacteria inside host immune cells in patients (Schwab *et al.*, 2014). While in zebrafish, neutrophils did not have an important role in acute virulence (Mesureur *et al.*, 2017), these findings together provide a paradigm changing view on the infection mechanism of these bacteria.

For these reasons, we adopted the zebrafish embryo model to unveil early stages of innate immune response of macrophages to Bcc bacteria that were grown in different pre conditions they might encounter in the lung environment, potentially predisposing the bacteria to the production of virulence factors. Therefore, in this project I analysed the influence of pre-incubation of bacteria in high salt concentrations (0.4M NaCl) prior to injection in zebrafish embryos on the virulence of *B. cenocepacia*. Using this model, bacterial multiplication, host survival and *in vivo* interactions with host cells were analysed.

By combining *in vitro* and *in vivo* results, we expect to study the influence of the salt stress under two different levels of complexity, at culture media, where we could analyse the pure effect of NaCl on virulence factor production, and under the influence of immune system and other factors present on zebrafish.

For zebrafish infections, the conditions used in Brazil were adapted to the protocol available for this bioassay. The first difference was that we used *B. cenocepacia* K56-2 instead of J2315, as it was much more characterized in the zebrafish model, although virulence levels between these strains are similar (Vergunst *et al.*, 2010).

For zebrafish infections, we analysed the differences in virulence between static (Brazil) and shaking conditions (France) to grow the bacteria in combination with high

osmolarity conditions, but for all other experiments we used bacteria grown in cultures on a rotary shaker to adhere to the optimised protocol for zebrafish infections. Results of infection obtained with static cultures were not reproducible, and gave problems with injections.

Using fluorescence microscopy, we observed that *B. cenocepacia* is highly influenced when cultivated in presence of 0.4M NaCl. Results have shown that the bacteria increased in about 0.4um their length ($p \leq 0.001$) when cultivated in LB 0.4M NaCl compared to LB, which could indicate that the bacteria has not yet completed their division cycle in this medium. Using dead/live staining, it was further shown that the bacteria lost about 30% of viability ($p \leq 0.01$), and reduced growth in shaking and non-shaking conditions in high salt conditions compared to regular LB.

Modifications of bacterial size have been described during long-term colonization of CF patients with *B. cenocepacia*, with variations up to 2x (Coutinho *et al.*, 2011). Although data regarding the way each species regulates its size is still scarce, it has been recognized that size of cells is defined by a complex process, being a combination of classical (as time of growth, for example) and molecular mechanisms, as cell cycle, replication of DNA and cell division (Chien *et al*., 2012; Willis and Huang, 2017).

It has been described that some prokaryotes have mechanisms that enable the survival under conditions of high osmotic pressure. Halobacteria, for example, that belong to Archaea domain, is classically very well adapted to salt environments and can deeply change its structure when under stress. For other groups belonging to the Bacteria, an equivalent mechanism also exists, and although it is not that as sophisticated it allows the osmolarity rates control of low mass molecules in the cytoplasm (Sleator and Hill, 2002). Although we used the same protocol to prepare the LB and LB 0.4M NaCl inocula to inject zebrafish, and the inoculum was corrected based on the number of live bacteria (CFU/mL), a large difference in bacterial CFU numbers was detected at T= 0 hpi in 5 randomly picked, individually sacrificed injected embryos (Fig 3, Chapter 2). It seemed the calculated injection dose of bacteria grown in high salt conditions (based on OD measurements and adjustment of live/death ratio) did not match the real injected dose calculated (based on CFU counts from embryos at T=0).

From microscopic observations it was apparent that cultures of K56-2 grown in high salt concentration formed small aggregates in liquid media that might have affected the injection volume. To be able to compare properly these conditions, further experiments would be necessary to standardize the conditions.

Based on the results obtained, it was not possible to dray any firm conclusion about NaCl effect on virulence on *B. cenocepacia*, but the results suggest that salt stress prior to injection does not significantly affect bacterial virulence in zebrafish. We cannot exclude that the bacteria rapidly adapt to the intracellular macrophage environment after their injection, and change their transcriptome, something that might also happen in human infections.

We have further tested the effect of pre-growth in ASMDM medium, that has been developed by Fung and co-workers to closely mimic the composition of CF sputum (Fung *et al*., 2010). ASMDM has previously been used to promote *P. aeruginosa* growth (Fung *et al.,* 2010), and the global transcription profile of *P. aeruginosa* in different growth phases was determined.

Results showed genes up (iron-acquisition and assimilatory nitrate reductase) and down (QS-genes and T3SS) regulated, and the global transcription profiles are compatible with the iron deficient and microaerophilic/anaerobic environment observed in CF lungs (Fung *et al.*, 2010). It has also been used by other researchers in infections of A549 carcinoma monolayers cells to study *B. cenocepacia* infection (Wijers *et al.*, 2016). In this stidy, A549 cells in presence of 60% of ASMDM showed to be more damaged after *B. cenocepacia* infections than cells grown in HAM's F12 complete medium, used as control (Wijers *et al.*, 2016).

Egg, mucin, sperm and other components used in ASMDM could elicit an inflammatory response in zebrafish. In order to exclude the possibility that the medium could contribute to virulence caused by increasing the inflammatory response, we included an additional control. The non-inoculated ASMDM media were centrifuged, and debris obtained were added to LB grown bacteria, dissolved in PBS to inject zebrafish embryos. The results have revealed no difference compared with bacterial inocula suspended in PBS alone. However, in contrast to the *in vitro* assays described above which were indicative of increased virulence, pre-cultivation of *B. cenocepacia* in ASMDM prior to zebrafish infection did not show a significant difference in virulence compared to bacteria grown in regular LB medium.

Cysteamine was the last condition tested *in vivo*. Cysteamine has been described to improve the phenotype of both CFTR F508del mice and CF patients, with benefits for CFTR functions in CF nasal epithelial cells (De Stefano *et al.*, 2014). This substance has also been shown to have antimicrobial effect against *P. aeruginosa* (Ferrari *et al*., 2017), *Mycobacterium abscessus*, and *B. cenocepacia* (Shrestha *et al.*, 2017).

It also ameliorated effectiveness of tobramycin, reduced viscoelasticity of mucus and acted against biofilms (Charrier *et al.*, 2014; Devereux *et al.*, 2015). Different from what was observed in prior studies, we could not detect an effect of the use of cysteamine during zebrafish infection on virulence, or as antimicrobial compound against *B. cenocepacia* after one hour of treatment.

We have tested this substance by adding it in the swimming water or injecting it in larvae simultaneously with the bacteria inoculum, yet no difference in virulence was observed by infection assays. Further, the expression of two pro-inflammatory cytokine genes, *cxcl8* and *il1b*, was not significantly affected by addition of cysteamine.

In summary, we have not found any evidence that pre-treatment of *B. cenocepacia* with high salt, ASMDM or cysteamine influenced the virulence of these bacteria in the zebrafish infection model. This could suggest that the changed transcriptome/proteome and the described effect on virulence factor production of these bacteria due to these conditions, as for instance reduced biofilm production and mucin adhesion due to high salt, do not affect virulence in this model. Indeed, the zebrafish model represents the intracellular, but critical stages of infection, and does not represent the early stages of colonization and invasion in the CF lungs. Therefore, analysis in other animal models that represent these stages during infection should be performed.

It could also indicate that the virulence of *B. cenocepacia* K56-2 is already so high, with infected embryos dying within 2 to 3 days after infection, that a further increase (AMSDM), or only slight reduction (NaCl, cysteamine) in virulence may not be significantly detected. Alternatively, the transcriptome changes induced by pre-culture conditions may be transient, and change rapidly after injection in the embryos, returning the bacteria to the "wildtype" stage.

A more direct approach may be to use bacterial mutants in virulence factors that have been shown to be affected under the here analysed growth conditions (high salt, ASMDM and cysteamine), and directly analyse an effect on virulence in the zebrafish, and other infection models.

Our results suggest that the analysed conditions do not have a significant effect on the ability of the bacteria to be phagocytosed, to replicate, and to cause a strong pro-inflammatory host response. Based on our recent finding that the bacterial factor AFC, which has antifungal activity, is critical for acute infection in zebrafish larvae, it would be interesting to determine whether the tested medium conditions have any effect on the expression of the regulator shvR or on the afc operon (Gomes *et al.*, 2018).

3. Final considerations

Our *in vitro* assays have demonstrated that, during stationary phase, *B. cenocepacia* produce more virulence factors, as shown for biofilms, ability to adhere to mucin and competitive assays. A positive modulation of virulence factor production was triggered in presence of NaCl and NAC at the concentration of 0.4M NaCl and 1g/L NAC, when they were combined in LB. In contrast to this, NaCl and NAC used individually reduced the virulence factor production. By exoproteome analysis, we found BipC, an effector protein related to secretion system, in supernatants of *B. cenocepacia* obtained from LB media. By *in vivo* assays done in zebrafish, the pre-treatment with NaCl, ASMDM and with cysteamine have shown to not affect *B. cenocepacia* virulence, pro-inflammatory cytokine production, and the infection progress, as detected by real time analysis. Zebrafish injected with cysteamine have not shown differences in comparison with control embryos.

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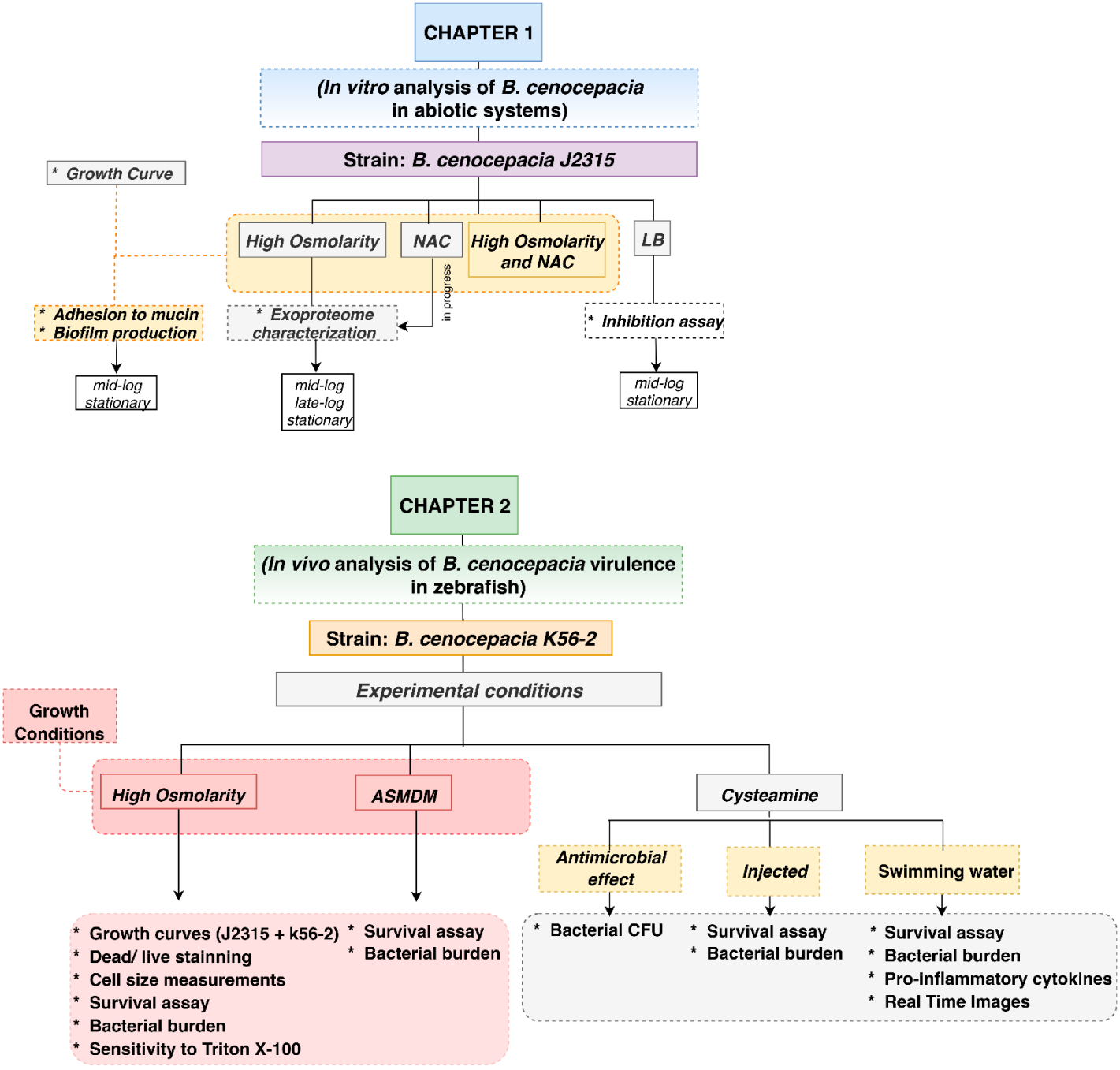
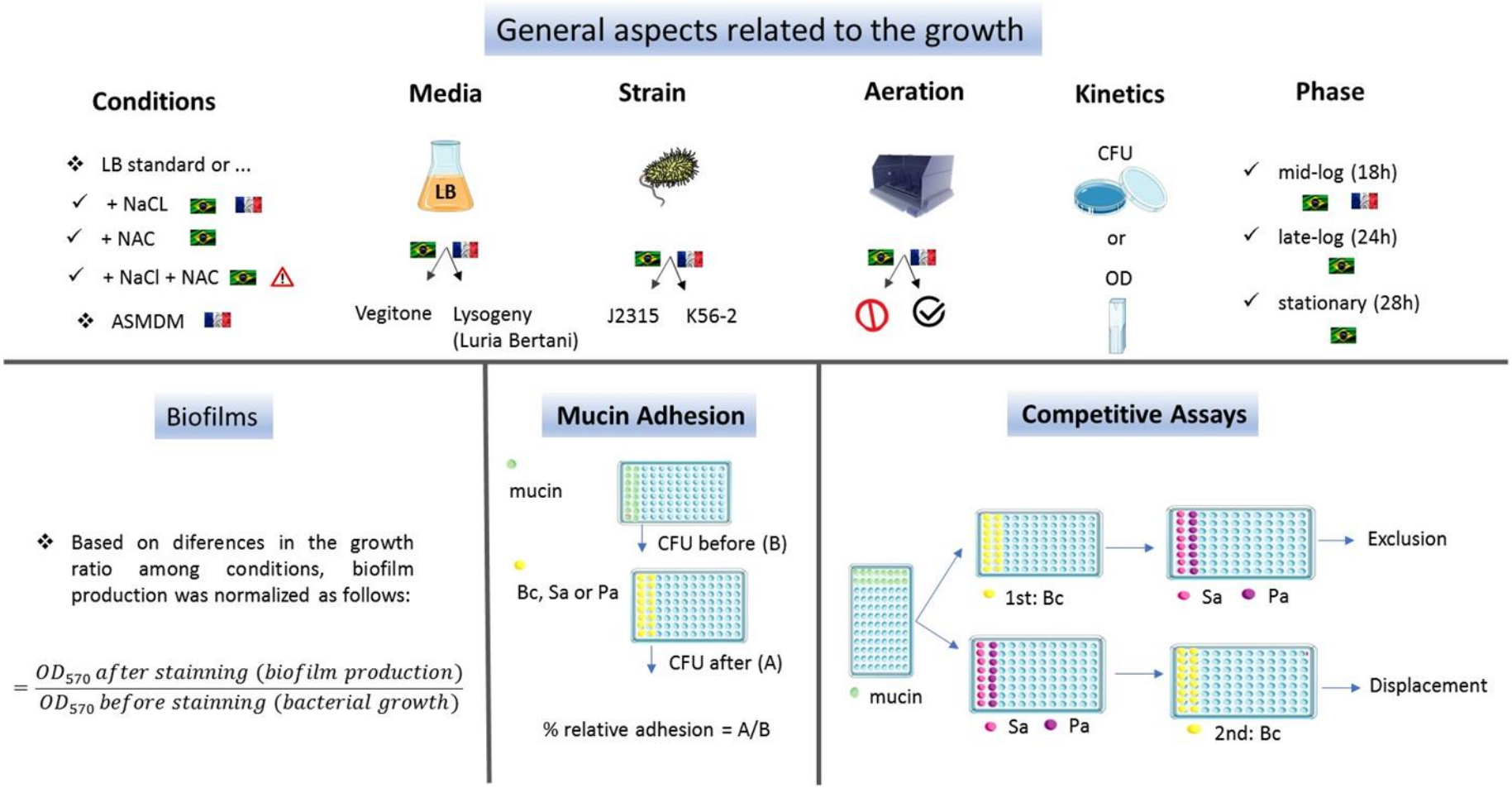
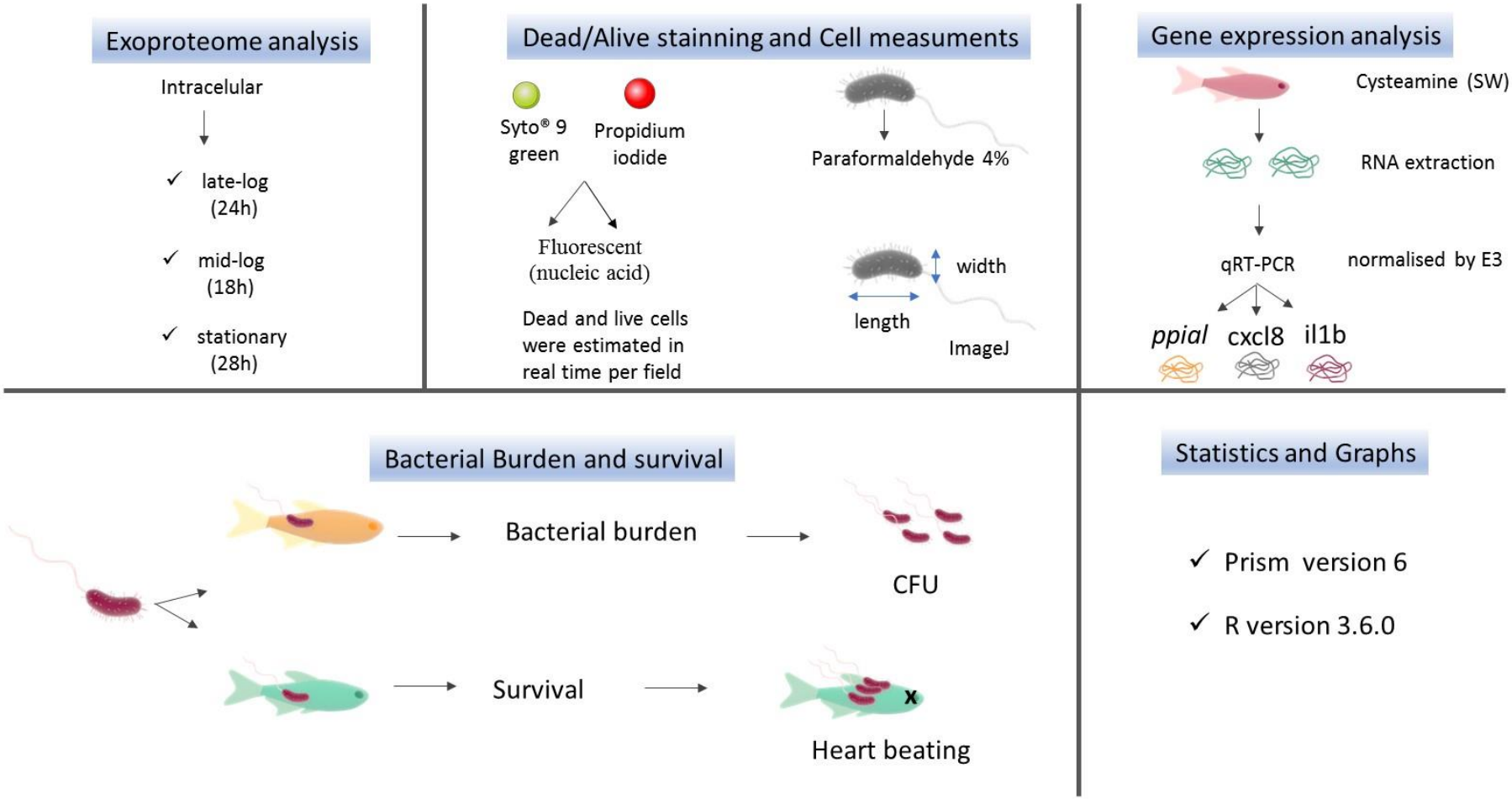
APPENDIX 1 - Methodology overview



APPENDIX 2 - Didactic overview of experiments



APPENDIX 2 - Didactic overview of experiments



APPENDIX 3 - Paper submitted

Title: *Are Burkholderia cenocepacia* virulence factors modulated in response to *in vitro* conditions similar to cystic fibrosis lungs?

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Abstract: Cystic fibrosis (CF) is a genetic disease that mainly affect the lungs. Recurrent bacterial infections are frequent and compromise patients quality of life. N-acetylcysteine (NAC) and Hypertonic Saline (HS) are some of complementary treatments available, but with little data about their effect on pathogen. Within this scenario, we investigated if the addition of these compounds, individually or combined, in the bacterial culture media would interfere, *in vitro*, with *B. cenocepacia* virulence factor production. To simulate the effect NAC and HS, we complemented LB broth with 0.4M NaCl or 1.0 g/L NAC, assessing for biofilm production and mucin adhesion. Also, we carried out competition assays between *B. cenocepacia* and *S. aureus* or *P. aeruginosa* in mucin to simulate the environment of CF lungs polymicrobial infections. These conditions were examined in two bacterial growth phases: mid-log and stationary. Results obtained indicate lower *B. cenocepacia* biofilm production in presence of NaCl and NAC, but when these compounds were combined, during the mid-LOG phase, more *B. cenocepacia* biofilm production was detected ($p \leq 0.01$). The adhesion to mucin in LB complemented with NaCl (0.4M) or N-acetylcysteamine (1.0 g/L) also had shown to be negatively affected in *B. cenocepacia*, except during stationary phase. In this case, presence of both compounds in LB showed an increase in mucin adhesion rate. We observed that *B. cenocepacia* showed less biofilms and low mucin adhesion rate than *S. aureus* and *P. aeruginosa*. In inhibition assays, *B. cenocepacia* excluded either *S. aureus* (79,39% and 75,20%) or *P. aeruginosa* (91,92% and 96,4%), during mid-LOG and stationary phase, respectively. *B. cenocepacia* was also able to displace *S. aureus* with 66,69% (mid-LOG) and 46,63% (stationary phase). The displacement of *P. aeruginosa* ranged from 14,99%, during mid-LOG, to 93,48%, at stationary phase, and it was greatly affected by the growth phase. In conclusion, results showed that NaCl (0.4M) combined to N-acetylcysteamine increase, *in vitro*, biofilm and mucin adhesion. Pathogen inhibition assays demonstrated, *B. cenocepacia* ability to exclude and to displace either *S. aureus* and *P. aeruginosa* from mucin, and that *B. cenocepacia* growth phase can highly influence interactions with *P. aeruginosa*. Currently, most investigations have focused on the efficacy and safety of different treatments associated to the host, and few emphasis is given to their effect on bacteria. In conclusion, *B. cenocepacia has* showed to increase virulence factor production in presence of both NAC and NaCl, but when they were individually tested, a negative effect was observed, with lower biofilm production and mucin adhesion. *B. cenocepacia* showed a strong ability to exclude and displace either *P. aeruginosa* and *S. aureus*. This study expands the knowledge about CF infections including interesting evidences of interactions among CF pathogens. Results showed will improve knowledge about pathogens during CF infections, and could help to trace new strategies of treatment.

Keywords: Cystic fibrosis, Hipertonic Saline, N-acetylcysteine, *B. cenocepacia*, mixed infections.

Introduction

Cystic fibrosis (CF) is a genetic disease related to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which originates faulty CFTR proteins (Rommens *et al.*, 1989; Gadsby *et al.*, 2006). CFTR is an ion channel present in

all cellular types (Hug *et al.*, 2003; Quinton, 2007). Dysregulations associated to CFTR mal-folded proteins can affect the airway surface liquid thickness, modify the composition of mucous and impairs the efficient removal of inhaled pathogens (Knowles and Boucher, 2002).

Patients experience during their life recurrent respiratory infections, which are defined as the major cause of death in CF (Ciofu *et al.*, 2013). During first years of life, CF patients are colonized with *Staphylococcus aureus* and *Haemophilus influenza*; after, *Pseudomonas aeruginosa* becomes the most frequently isolated bacterial specie (Harrison, 2007). Despite *B. cenocepacia* is not recognized among the most frequent pathogens associated to CF, it causes severe and difficult to treat infections in CF patients during adulthood (Lipuma *et al.*, 1990; Govan *et al.*, 1993; Jones *et al.*, 2004; Mahenthiralingam *et al.*, 2005; Harrison, 2007). The Edinburgh-Toronto is a *B. cenocepacia* epidemic clone (ET-12) associated with a fatal syndrome known as syndrome cepacia (Mahenthiralingam *et al.*, 2005). Important molecules associated with communication, adhesion, invasion, survival and activation of immune responses have already been identified in ET-12 strains (Mahenthiralingam *et al.*, 2005; Loutet and Valvano, 2010; Mcclean *et al.*, 2016). Recently, polymicrobial communities in pulmonary infections started to recognized in CF (Sibley *et al.*, 2006; Price *et al.*, 2013).

For a long time, hypertonic saline (HS) and N-acetylcysteine (NAC) are been suggested as complementary strategies of CF treatment in periods of acute exacerbations (Reas, 1964; Williams *et al.*, 2010), and represent a factor that can transitorily affect the surface of pulmonary epithelia. As they are been used for a long time to treat many pulmonary diseases, it represents a big advantage as a lot of knowledge is accumulated about the safety associated to their usage (Tirouvanziam *et al.*, 2006; Williams *et al.*, 2010). HS have shown to restore ASL hydration levels, to break ionic bounds in the mucus, and release negative charge, alleviating viscosity. It also improves expiration, and consequently, reduces inflammation and edema (Dentice *et al.*, 2016; Nenna *et al.*, 2017).

NAC is used as an expectorant agent with mucolytic properties (Ehre *et al.*, 2014). It stimulates the ciliary action and the vagal gastro-pulmonary reflex, and the consequent mucus removal from the upper airways (Ziment, 1988). It can be combined with antibiotics, and have shown good results (Hussain *et al.*, 2015). Recently, it was found to act against biofilms (Blasi *et al.*, 2016), and to restore intracellular levels glutathione, a natural antioxidant found to be decreased in CF (Conrad *et al.*, 2015). Despite all highlighted benefits (Dauletbaev *et al.*, 2009; Conrad *et al.*, 2015), clinical trials are still inconclusive about its efficacy in CF (Dauletbaev *et al.*, 2009; Nash *et al.*, 2009).

Little is known about the effect of NAC and HS on virulence traits of *B. cenocepacia*. Thus, it would be interesting to known how *B. cenocepacia* reacts, *in vitro*, in presence of these conditions. The aim of this study is to test virulence factor production by *B. cenocepacia* J2315 in presence of NAC at 1g/L and sub-inhibitory doses of NaCl (0.4M). The benefit of these pharmacological compounds are being investigated as they started to be used as complementary treatment during pulmonary exacerbations in CF patients. This study has, for the first time, described the combined effect of NAC and high concentrations of NaCl on *B. cenocepacia* virulence factor production. To address these questions we evaluated the tolerability of this bacterium to increased concentrations of NaCl and NAC by growth curves, and then, analysed biofilm production and mucin adhesion to understand the impact of these conditions on *B. cenocepacia* behavior *in vitro*, during mid-LOG and stationary phase. As a reference, to compare with *B. cenocepacia*, we used *S. aureus* and *P. aeruginosa*, that represent classical CF pathogens. *B. cenocepacia* was challenged with *S. aureus* and *P. aeruginosa* in inhibition assays, to simulate another host condition described *in vivo* during polymicrobial infections.

Material and Methods

Bacterial strains, quality control and stocks

B.cenocepacia J2315 is a highly transmissible epidemic clone that was isolated for the first time in Edinburgh, in the United Kingdom (ET12) (Govan *et al.*, 1993). It was kindly provided by Dr. Elizabeth de Andrade Marques (Department of Microbiology, Immunology and Parasitology of UERJ). *S. aureus* ATCC 25923 *and P. aeruginosa* ATCC 27853 from our laboratory collection were used in this study as well. Stocks were prepared from pure colonies obtained on blood agar grown at 36 ± 1 ° C for 24 to 48 hours. They were kept cryopreserved on Tripticasein Soy Broth (TSB) with glycerol (15%) at -20 ± 1 ° C. *B. cenocepacia* J2315, *S. aureus* ATCC 25923 *and P. aeruginosa* ATCC 2785 were used to access biofilm production, adhesion to mucin, and on inhibition assays. Also, we used *B. cenocepacia* J2315 to obtain growth curves. All strains had previously been identified based on molecular tests and checked to accomplish with standard biochemical and physiological tests expected for their profile (Jorgensen and Pfaller, 2015; Clsi, 2019).

Growth conditions and culture media

For cultivation of bacteria, strains were routinely obtained from cryotubes. They were pre-activated in LB Vegitone broth and incubated at 36 ± 1 °C with aeration (9 x g, 24-48h). After bacterial growth, suspensions were centrifuged (27.000 x g for 5 min), and pelleted cells were washed and resuspended in saline solution (0.9%). A nephelometer was used to obtain cell density of 0.5 in MacFarland scale. Then, the suspensions were diluted to a concentration of approximately 10^7 CFU/mL which was used in specific media. For experiments, growth was performed at the same temperatures but, instead of aeration, static cultures were used. At first, we used an overnight culture to grow bacteria to obtain growth curve. After, the incubation time was adjusted in each experiment according to the bacterial growth phases. LB Vegitone Broth was complemented with (i) NaCl to obtain LB 0.1M, 0.3M, 0.4M and 0.5M NaCl, with (ii) NAC to obtain 0,5 g/L, 1,0g/L or 2,0g/L NAC, or (iii) with NaCl and NAC to obtain LB 0.4M NaCl and 1,0g/L NAC. NAC was prepared immediately before use, filtrated in 0.22µm membranes, and added to the autoclaved medias. Culture media used were used as stated in each section. NAC was prepared immediately before use, filtrated in 0.22µm membranes, and added to the autoclaved medias to obtain LB + NAC at 0.5 g/L, 1.0 g/L and 2.0 g/L.

Growth curve

To obtain growth curves, we started with an inocula with 10^7 CFU/ mL in LB. Sterile 96-well polystyrene plates with flat bottom were used to obtain the OD_{600}. Flasks with the bacterial culture were kept without aeration in the incubator for 48h at 36 $\pm$1ºC. At first, *B. cenocepacia* J2315 growth phases were defined based on OD_{600} and CFU counting at the time points 0h, 5h, 10h, 20h, 25h, 30h, 35h, 45h and 48h. CFUs were expressed in $\log_{10}$ (CFU/mL) in one experiment performed.

After, we tested LB with NAC and NaCl, in all conditions described in the previous section to check if complementing LB with increasing concentrations of NaCl and NAC it could affect bacterial growth. We started with 10^7 CFU/ mL, and we used the OD_{600} to compare the bacterial growth. Non-inoculated LB (or LB complemented with

different conditions) was used as a negative control. Microplates were incubated in the microplate reader for 22h at 36 ± 1°C, and the equipment was programmed for readings every hour, with prior shaking of 15s. Each condition was performed at least in triplicates in individual experiments, and it was repeated once. The results expressed are means obtained in one representative experiment.

In vitro biofilm production

To assess the *B. cenocepacia* ability to produce biofilm, sterile 96-well polystyrene plates with flat bottom were used. We started from 10^7 CFU/ mL suspensions, and 200µl were delivered into each well. Microplates were incubated at 36 ± 1° C, without shaking. Culture media used for biofilm detection were LB, LB 0.4M NaCl, LB with 1.0 g/L NAC; LB 0.4M NaCl + 1g/L NAC. *B. cenocepacia* was evaluated during mid-log and stationary phase. For comparisons, *S. aureus* and *P. aeruginosa* were grew for 24h on the same culture media, as these species are known as biofilm-producers related to CF.

The ability of *B. cenocepacia* to produce biofilm was quantified according to Tendolkar and colleagues (Tendolkar *et al.*, 2004), with the following modifications. As we observed differences in bacterial growth in the modified culture media, we normalized the biofilm production based on the growth. After incubation, OD was measured at λ = 570nm in SpectraMax Plus 384 Microplate Reader (Molecular Devices). At first, OD was obtained before removal of the planktonic cells, to assess growth, previous to the staining of the microplates with violet crystal. After staining, individual tips were used to homogenize biofilms residues stained onto the walls of each single well, and then, we obtained a second OD_{570} value after the treatment with violet crystal, to measure biofilm produced. To normalize biofilm production, we divided final OD obtained after staining (indicative of biofilm production) by the OD registered before staining (bacterial growth). The final values obtained were used to construct the dox plot. Non-inoculated wells containing LB broth were used as a control. Each assay was performed twice, with seven biological replicates. Representative results of one of the experiments are presented. (O'toole and Kolter, 1998).

In vitro adhesion to mucin

The procedure described by Valeriano and co-workers (Valeriano *et al.*, 2014) was used to assess adhesion of *B. cenocepacia* J2315 to mucin. To evaluate the ability to adhere to mucin, *B. cenocepacia* was grown in LB, LB 0.4M NaCl, LB 1.0g/L NAC and LB 0.4M NaCl + 1.0g/L NAC. Some modifications indicated below were based on a previous work developed by (Sanchez *et al.*, 2010). *B. cenocepacia* was grown for 18h (mid- logarithimic) and 28h (Stationary) phase to explore differences in adhesion referent to the growth phase. *S. aureus* and *P. aeruginosa* were included for comparisons with *B. cenocepacia*, as they are commonly associated to CF infections. In this case, these bacteria were grown for 24h and 28h to compare with *B. cenocepacia* in the mid-logarithimic and stationary phase, respectively.

Approximately 100µL of mucin from porcine stomach Type II partially purified (1mg/ml, Sigma- Aldrich ®) dissolved in phosphate-buffered saline 1x (PBS 1x) was added to each well of sterile 96-well plates. Polystyrene flat bottom microplates used were incubated for 1h at 36 ± 1° C. They were then let overnight to immobilize at 4° C. Wells were then washed twice with PBS 1x to remove non-bound mucin. Then, 100µL of bovine serum albumin (BSA at 20 mg/mL in water for injection) were used to block

non-bound sites on the wells and incubated for an additional 2h at 4° C. After this, plates were carefully washed twice with 200µL of PBS 1x. Overnight cultures of bacterial cells at exponential (18h) or stationary (28h) phase were washed once with 0.9% saline solution, adjusted to 10^7 CFU/mL in PBS 1x and from this suspension, 100 µL were inoculated on each well containing mucin for 1h at 36 ± 1° C. It was followed by five washing steps with citrate buffer (200 µL), used to remove non-adherent bacteria. An additional volume of 200 µL of Triton X-100 solution at 0.5% (v/v) was used to detach bacteria from the wells, and viable bacterial counts were determined by plating in *Mueller Hinton Agar* (MHA), and expressed as CFU/mL. Percentage of adhesion was obtained according to Collado and co-workers (Collado *et al.*, 2008) based on the formula described below. Two independent experiments were done, and graphs were constructed with data obtained in one representative experiment.

$$\% \text{ Relative Adhesion} = \frac{\text{CFU/ml after adhesion}}{\text{CFU/ml before adhesion}} \times 100$$

In vitro exclusion and displacement assay

To evaluate the behavior of *B. cenocepacia* J2315 in presence of *S. aureus* and *P. aeruginosa*, the ability of *B. cenocepacia* to exclude and displace these other species was investigated. Briefly, this assay was based on (Collado *et al.*, 2008), adapted (Valeriano *et al.*, 2014) and modified to include minor changes, as described here. LB Vegitone Broth was used to grow strains, at the same conditions described above for adhesion assay. Microplates were prepared as described in the previous session. After wells were whashed with PBS to remove non-bound BSA, *B. cenocepacia* J2315 was incubated to adhere to mucin, the same way described in the mucin adhesion assay. After 1h at 36 ± 1° C, the suspension was completely removed, and wells were washed twice with PBS 1x. Then, a volume of 100 µL of the second suspension (containing *S. aureus* or *P. aeruginosa*), was inoculated and incubated at 36 ± 1° C for additional 1h for the exclusion assay. Non-adhered bacteria were removed by five cycles of washing with 0.9% NaCl. Each well was treated with 200 µL of Triton X-100 to detach adherent bacteria, and by the same way described before, CFU was obtained by plating. Inversely, for displacement assays, plates were first incubated with *S. aureus* or *P. aeruginosa*, and then with *B. cenocepacia*. Experimental quadruplicates were prepared for each condition, used to obtain the media and from this, we calculated percentage of exclusion and displacement. Each experiment was repeated once. Percentage of inhibition was calculated based on (Collado *et al.*, 2008), according to the following formula described below. Averages and standard deviation were calculated and used to express results obtained.

$$\%\text{inhibition} = \frac{(\%\text{ adherence of pathogen}) - (\%\text{ adherence of pathogen with probiotic})}{\%\text{ adherence of pathogen}} \times 100$$

Statistical analysis

Statistical analysis was performed using R version 3.6.0 and charts were constructed using Prism Version 6. For growth curves, trend values with 95% confidence intervals were estimated by Ordinary Least Square (OLS) in order to compare them. The Wilcoxon Test was performed to evaluate the difference between biofilm production and mucin adhesion assay median.

The median value of individual bacterial species in each different culture media for biofilm assay and mucin adhesion were compared in relation to the control (LB). In *B. cenocepacia*, growth phases (mid- log and stationary) were also compared. Although not indicated in the graphics, different species (*B. cenocepacia* during mid-log and stationary phase, *P. aeruginosa* and *S. aureus*) were compared in each culture medium, statistically analysed and results obtained are described in each section. Additionally, for mucin adhesion assay, both biological replicas were compared and only one representative experiment is shown. For *S. aureus* and *P. aeruginosa* inhibition assays, averages of percentages obtained in two independent experiments $\pm$ SD were showed.

Results

At higher concentrations, NaCl and NAC slowed down *B. cenocepacia* J2315 growth kinetics

During treatment, NAC and HS can be transitorily present in the lungs of CF patients. To investigate virulence factor production by *B. cenocepacia*, we used LB complemented with NAC and NaCl to evaluate if the presence of these substances would impair *B. cenocepacia* growth. Based on CFU and OD_{600}, we obtained *B. cenocepacia* J2315 growth curves on LB (Figure 1a). Mid-log, late-log and stationary phase were reached at 18h, 24h and 28h, respectively (Figure 1a). Absorbance at OD_{600} was also used to obtain growth curves in LB + X NaCl (X= 0.1M, 0.3M, 0.4M and 0.5M) and LB + Y NAC (Y= 0.5g/L, 1g/L or 2.0 g/L). Results showed on Figure 1b indicate that, any of the conditions tested exerted a complete inhibition against J2315 growth. At 0.1M NaCl, 0.5g/L and 1.0 g/L of NAC, bacterial growth was similar to LB. Nevertheless, in LB + NaCl 0.3M, and more accentuated in LB 0.4M or 0.5M NaCl and in LB 2.0g/L NAC, growth was slower than the control with LB. Time trends of *B. cenocepacia* J2315 growth obtained for each individual growth curve with a confidence interval of 95% (Table 1) confirmed differences showed in Figure 1b. In Figure 1b it is possible to see that bacteria usually take 6h to start growing in most of culture media tested. However, in presence of LB 0.4M or 0.5M NaCl and in LB 2.0g/L NAC, bacteria take more time to start log phase (11h) and have a less pronounced growth, expressed by means of OD.

B. cenocepacia biofilm production increases at stationary phases and it is lower in presence of LB 0.4M NaCl or 1g/L NAC

We assessed the *B. cenocepacia* ability to produce biofilm in presence of NaCl and NAC at mid-log and stationary phase. For comparisons with *B. cenocepacia*, we grew *S. aureus* and *P. aeruginosa* in overnight cultures on the same culture media, as these species are biofilm-producers related to CF. As can be seen in Figure 2, the ability to produce biofilm of *P. aeruginosa* was the strongest, while *S. aureus* was moderate and *B. cenocepacia* was the lowest (Figure 2a, b and c). Across species, in all comparisons done between similar condition, for instance LB x LB, the p value was $p \leq 0.0001$.

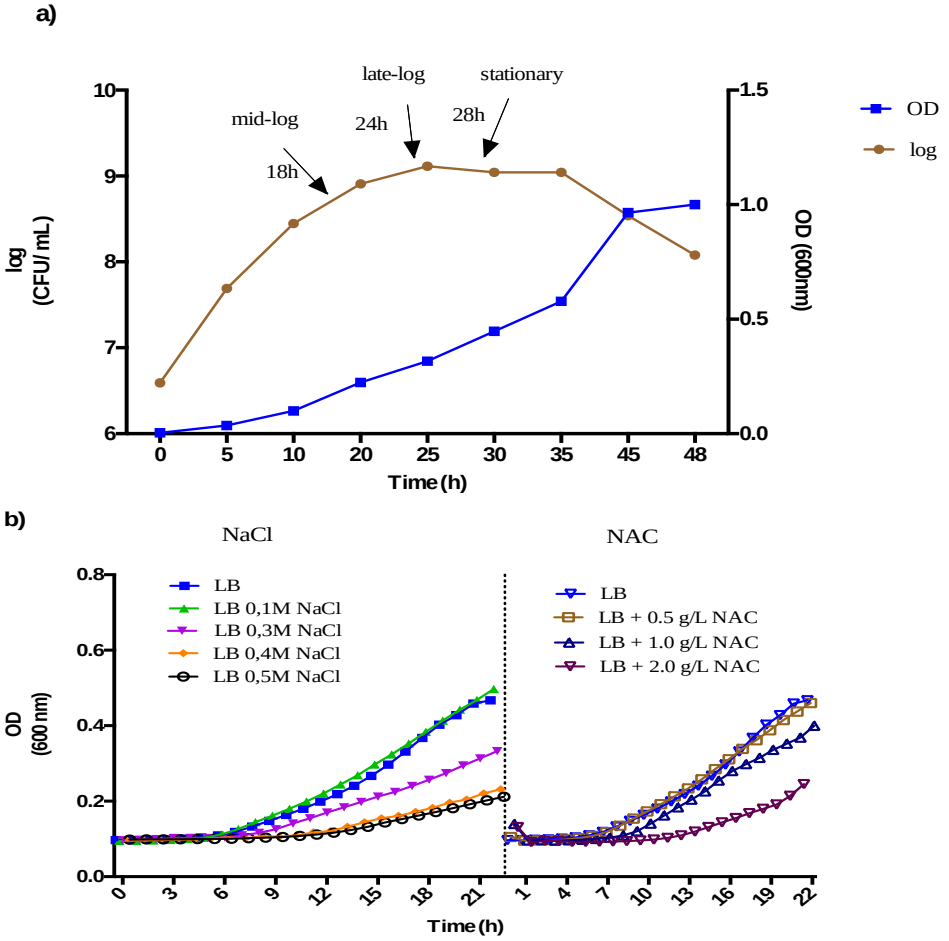

Figure 1: *B. cenocepacia* J2315 ability to grow in different culture media. In **(a),** growth curve obtained in LB media based on OD (600nm) and log (CFU/ml). Mid- log (18h), late- log (24h) and stationary (28h) phases are indicated. In (**b**), bacteria were cultivated in LB complemented with different concentrations of NaCl (0.1M, 0.3M, 0.4M and 0.5M) and NAC (0.5, 1.0 and 2.0 g /L). Growth curves were obtained up to 22h and expressed as OD (600nm).

Table 1: Time trends of *B. cenocepacia* J2315 growth in different culture media. Time trends were obtained for each culture media based on growth curves calculated (OD_{600}). The 95% confidence intervals estimated by Ordinary Least Square (OLS) are also shown.

Culture Media	Time Trend (OD600)	CI Lower (95%)	CI Upper (95%)
LB	0.0181	0.0156	0.0207
LB 0.1M NaCl	0.0194	0.0171	0.0216
LB 0.3M NaCl	0.0111	0.0096	0.0125
LB 0.4M NaCl	0.0061	5	0.0071
LB 0.5M NaCl	0.0052	0.0042	0.0061
LB	0.0181	0.0156	0.0207
LB 0.5 g/L NAC	0.0178	0.0157	0.02
LB 1.0 g/L NAC	0.0144	12	0.0168
LB 2.0 g/L NAC	0.0069	0.0047	9

The addition of 0.4M NaCl in the media used to cultivate *B.cenocepacia* and *P. aeruginosa* affected the biofilm production of these bacteria, as both *B. cenocepacia* ($p \leq 0.001$, during mid-log phase) and *P. aeruginosa* ($p \leq 0.01$) biofilms were negatively affected (Figure 2a, b). However, no difference was observed for *S. aureus* in comparison with the control ($p = 0.11$, Figure 2c).

In respect to *B. cenocepacia* growth phases, more biofilm was produced during stationary phase (Figure 2a). In all pair-pair comparisons, the significance obtained between growth phases with the same media was $p \leq 0.0001$ (Figure 2a). The only exception was verified when either LB 0.4M NaCl and NAC (1g/L) were added to LB, as no difference in biofilm production was detected between *B. cenocepacia* mid-log and stationary phases ($p = 0.42$, Figure 2a). However, *B. cenocepacia* biofilm in presence of 0.4M NaCl and NAC (1g/L) showed greater biofilm levels on mid- log phase in relation to the LB used as control ($p \leq 0.01$, Figure 2a).

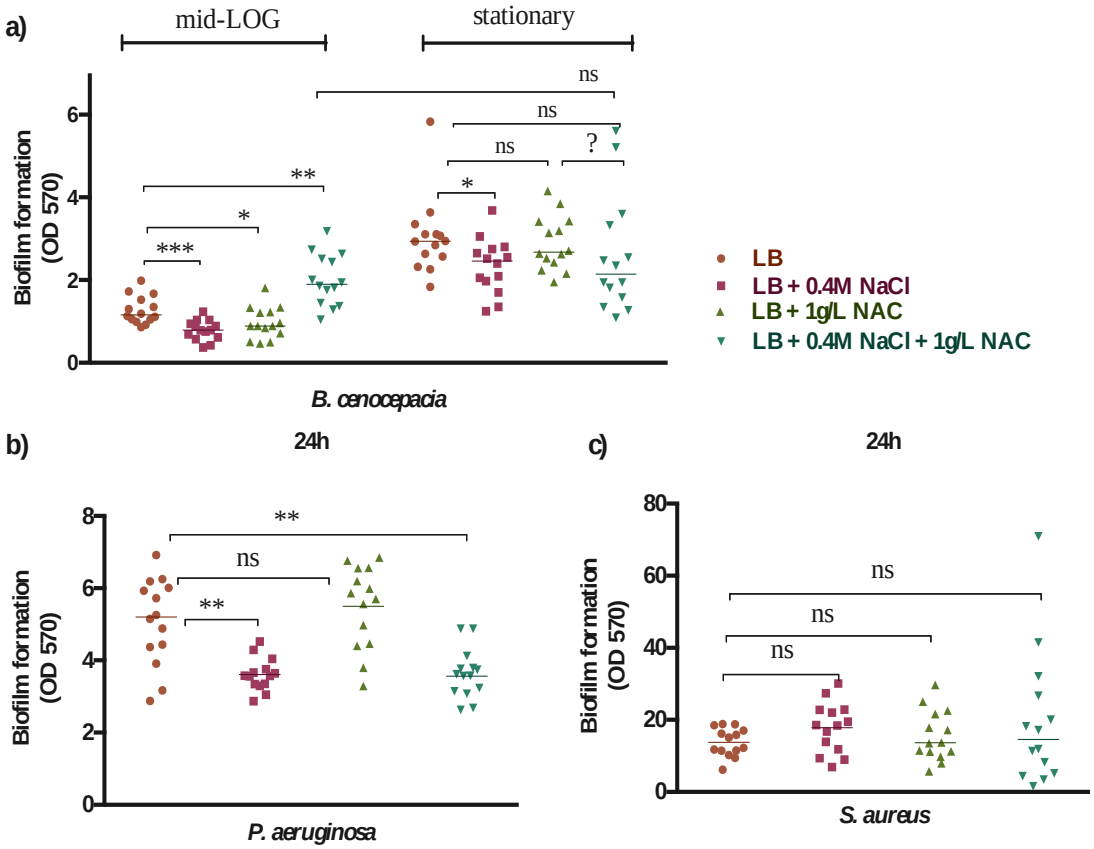

Figure 2: Biofilm production in different growth phases. On the top, *B. cenocepacia* scatter charts in mid-logarithmic (mid-log, at 18h) and Stationary (28h) growth phases **(a)**; on the bottom, *P. aeruginosa* **(b)** *and S. aureus* **(c)** scatter chart representing biofilm formation after 24h of growth. Each condition used to grow bacteria is represented in a vertical line, and each symbol represents individual values of experimental (n=7) and biological replicates (n=2). Horizontal bars represent medians obtained. Groups are represented as follows: LB medium (control, brown, dots) supplemented with 0.4M NaCl (pink, squares), 1.0 g/L NAC (light green, regular triangles), or both (0.4M NaCl + 1.0 g/L NAC, dark green, inverted triangles). Each well was inoculated with 200µL from a suspension containing approximately 10^7 CFUs/mL. Biofilms were revealed with crystal violet. On the y-axis, ratio of absorbances obtained before and after staining with violet

crystal with readings at OD 570nm; on the x-, microorganisms are indicated. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ and ns: not significant. See material and methods for statistical analyses.

The ability of *B. cenocepacia* to adhere to mucin increases during stationary phase and is reduced in presence of either 0.4M NaCl or 1g/L NAC

The adhesion to mucin was investigated here as another virulence factor produced by *B. cenocepacia*. We found *B. cenocepacia* had a higher adhesion to mucin during the stationary in comparison with mid-log phase (Figure 3a). Adhesion to mucin was lower in presence of either 0.4M NaCL or 1g/L NAC, except during stationary phase in which it was found to be equal to the control (LB). Only when these compounds were combined, an increase in the adhesion to mucin was observed (Figure 3a). *S. aureus* and *P. aeruginosa* showed the highest adhesion to mucin rates after 24h of growth in comparison with *B. cenocepacia* in both phases.

In Figure 3b, between *B. cenocepacia* mid-log and stationary phase, *P. aeruginosa* showed an expressive difference in its ability to adhere to mucin. We have used the same culture for both experiments, with only four hours of difference between experiments (Figure 3b). We suppose during the time -between processing (4h), *P. aeruginosa* reached a critical phase in which bacteria varied deeply in its ability to adhere to mucin. Although these differences showed on graphics, no statistical difference was observed, considering $\alpha=0.05$, probably due to the limited sample size, and the consequent low statistical power of the analysis.

Inhibition of *S. aureus* and *P. aeruginosa* by *B. cenocepacia*

Results obtained in inhibitory assays are showed in Table 2. The exclusion assay measures how much a *B. cenocepacia* associated to mucin impairs the adherence of a 2nd pathogen (*S. aureus* or *P. aeruginosa*). In the displacement assay, the pathogens are already adhered to mucin when *B. cenocepacia* is included in the system. Then the *B. cenocepacia* ability to remove (or displace) pathogens previously adhered to mucin is measured.

B. cenocepacia has showed ability to exclude and displace *S. aureus* and *P. aeruginosa* strains at both growth phase (mid-log and stationary). *S*trong differences to displace *P. aeruginosa* according to the growth phases were detected: although displacement at mid- log very low (14.99$\pm$2.55), it showed to be highly accentuated during stationary phase (93,48$\pm$0.37). In an opposite way, *B. cenocepacia* showed better ability to displace *S. aureus* in earlier steps, at mid- log phase (66.69$\pm$2.08), but it was kept during stationary phase (46.63$\pm$29.29). In the exclusion assays, *B. cenocepacia* showed similar ratios against both, *S. aureus* and *P. aeruginosa* strains, and among growth phases tested too. *B. cenocepacia* excluded *S. aureus* with 79.39$\pm$15.68 or 75.20$\pm$29.44 and *P. aeruginosa* with 91.92$\pm$0.66 or 96.4$\pm$0.29, during mid-LOG and stationary phases, respectively.

Discussion

During stationary phase more biofilm production and mucin adhesion were detected in comparison with mid-log phase. In agreement to this, Saa and colleagues observed when they assessed *B. cenocepacia* J2315 by transcriptomic profiling, that upregulation of extracellular virulence factor genes is more pronounced during stationary

phase (Sass *et al.*, 2013). Also, more than 25% of the global transcripts was found to change in response to the stationary phase and under limited oxygen atmosphere or growth arrest as well (Sass *et al.*, 2013). These results could suggest that, during infection, bacteria does not start its virulence factor production immediately after getting assess to the pulmonary environment of the host, but instead, at first *B. cenocepacia* adapt and grow logarithmically to start the virulence factors synthesis.

Non-shaking cultures were used to grew bacteria to reduce the levels of oxygen, although traditionally *B. cenocepacia* is known as non-fermenting obligatory aerobic microorganism (Vandamme *et al.*, 2003). We decided to use this condition to get close to the CF lungs environment. The sputum interface is characterized by an oxygen gradient (Yang *et al.*, 2011) that can show very low rates especially within the mucus, that can reach 0% of oxygen (Yang *et al.*, 2011). A high occurrence of strict anaerobic bacteria is reported in association to the CF sputum, which is in agreement with an environment with reduced oxygen level. In some cases, the amount of strict anaerobic bacteria can be equivalent to the number of aerobic pathogens related to CF pulmonary infections (Tunney *et al.*, 2008). The growth curves obtained here endorsed the ability *B. cenocepacia* to growth, and even under non-shaking, J2315 started the logarithmic phase only after 6h it was inoculated, entering in the decline phase early, with only 35h. This is in harmony with the recent description of a novel low-oxygen-activated locus that enable *B. cenocepacia* survive in the absence of oxygen , in conditions similar to the CF lungs (Sass *et al.*, 2013).

The concentrations of NAC and NACl we used to cultivate bacteria were chosen based on reports found in the literature. For example, in a recent clinical trial, authors have defined a treatment using oral NAC at a dose of 120 mg twice per day (Skov *et al.*, 2015), showing an increase in lung function. Also, the concentrations practiced for *in vitro* studies with gram-negative bacteria usually employ NAC varying from 0.5 to 2.0 g/L (Blasi *et al.*, 2016).

Salt stress is also a condition naturally found to occur in CF patients, as 40%-50% more NaCl are detected in CF sputum or CF airway surface liquid (ASL) samples in comparison with healthy individuals, which show 7.4g/L of NaCl (Joris *et al.*, 1993; Grandjean Lapierre *et al.*, 2017). Besides, the use of HS (3-7%) to nebulize patients during treatment can increase much more the NaCl concentration. We cultivate bacteria at 0.4M NaCl (final concentration of 2.4%), which seemed to be a reasonable amount to start investigations as, to our knowledge, the bioavailability of NaCl after CF patients are nebulized with HS have not been reported yet. Thought, the NaCl concentration in patients under treatment with HS remains to be elucidated.

We seek for sub-inhibitory NaCl and NAC concentrations, as close as possible to the concentrations these substances reach in CF lungs. Growth curves obtained in LB media complemented with NaCl at 0.3M, 0.4M or 0.5M NaCl, and with NAC at 2g/L as well, showed a reduced growth rate and seemed to be a disadvantage for *B. cenocepacia*. Based on this, we settled the sub-inhibitory concentrations of these compounds and decided to select 0.4M NaCL and 1g/L NAC as sub-inhibitory concentrations to test *B. cenocepacia* virulence factor production without compromising too much the bacterial growth.

For the first time, we tested the effect of 1g/L NAC in a context with higher NaCl (0.4M). Both conditions were combined in the culture media to examine whether high NaCl levels expected to occur in CF lungs in association with NAC, could benefit CF patients by reducing *B. cenocepacia* virulence factor production. Either biofilm production and mucin adhesion demonstrated to be higher in presence of both compounds. This is the pioneer study that shows that *B. cenocepacia* under salt stress and

in presence of NAC increase virulence factor production. Preliminary results obtained here indicate that this effect must be further investigated deeply, as it could represent a big disadvantage for those thinking in the use of NAC as alternative treatment for CF patients.

At 0.4M NaCl, we observed that *B. cenocepacia* reduced both biofilm production and mucin adhesion. An opposite effect was observed by Tomich and Moher (2004) in a study developed with *B. cenocepacia*. Authors have suggested that high osmolarity (0.1M and 0.2M NaCl) have induced the adherence to the lower respiratory tract mediated by a cable pilus (*cbl*) (Tomich and Mohr, 2004). It had been also showed for *B. pseudomallei*, a closely related specie to *B. cenocepacia*, that neither flagella production nor biofilms are influenced in a NaCl range from 0.15 to 0.3M NaCl (Pumirat *et al.*, 2017). However, in presence of NaCl, bacteria experienced an adaptative change, enhancing plaque formation, heat and oxidative resistance (Pumirat *et al.*, 2017). Another study had showed similar results, with no effect of hypertonic saline 7% in *B. cenocepacia* mature biofilms (Narayanaswamy *et al.*, 2019).

Results obtained in LB 0.4M NaCl fit in a theory proposed by Williams and colleagues for *P. aeruginosa*, which suggests this bacteria vary its phenotype in osmolarity stress, as presence of HS. *P. aeruginosa* would change from mucoid to a non-mucoid phenotype associated to brand clinical symptoms, and then, the non-mucoid, more virulent and osmotically sensitive colonies would take place the niche previously occupied by the mucoid phenotype (Williams *et al.*, 2010).

The same way we have showed here, it is widely accepted that *B. cenocepacia* form biofilms *in vitro*. However, its clinical relevance during infections started to be argued in the last years, as *B. cenocepacia* were found *in vivo* in "micro colonies or as single cells within phagocytes or in mucus, and not in biofilm-like structures" (Sajjan *et al.*, 2008; Schwab *et al.*, 2014). It has been also demonstrated that *B. cenocepacia* interfere with essential steps of phagolysosome maturation and cellular trafficking (Abdulrahman *et al.*, 2011; Al-Khodor *et al.*, 2014; Valvano, 2015), and that it survives and replicates inside macrophages and epithelial cells (Valvano 2015). In agreement with clinical observations, *in vivo* evidences that intramacrophage stages are critical for *B. cenocepacia* were reported by Mesureur and colleagues (Mesureur *et al.*, 2017) using a zebrafish infection model. All those findings may provide a paradigm changing view on the infection mechanism of these bacteria.

For many pathogens, adhesins are mandatory for the pathogen during first stages of infection. They represent a special virulence factor that enable pathogens adhere to many sites, as intestinal and respiratory mucosa. Adhesion is indicative of higher virulence as well, as more mucin binding rates are associated to strains isolated from severe patients in which patient`s condition evolves to death (Sajjan *et al.*, 1992).

It could be argued that we used mucin from porcine stomach, and not from respiratory system to test mucin adhesion and competitive assays. It is worth to highlighting that it is both mucins are considered equivalents. For instance, mucin from porcine stomach was also used by others in a media developed to cultivate bacteria in conditions mimicking cystic fibrosis lung sputum (Sriramulu *et al.*, 2005). Another study showed evidences that CF (respiratory) and non-CF (intestinal) mucins are equivalents, as strains associated to the syndrome cepacia showed the same adhesion capability in both sources (Sajjan *et al.*, 1992).

In our study, we showed pioneering that even at low doses (1g/L) NAC reduced *B. cenocepacia* biofilm production and mucin adhesion. There is no previous report exploring the effect of this compound against *B. cenocepacia* mucin adherence. Activity against *B. cenocepacia* biofilm, in the other hand, had been explored in only one

published paper, in which authors found a similar effect when they tested NAC at higher concentrations (8g/L) (Pollini et al., 2018). NAC was found to inhibit two days-old biofilms and to diminish *B. cenocepacia* growh (Pollini et al., 2018). The antimicrobial effect of this substance against *P. aeruginosa* (Zhao and Liu, 2010) and other pathogens have also been reported (Olofsson *et al.*, 2003; Moon *et al.*, 2015; Abdel-Baky *et al.*, 2017). Moreover, a systematic review highlights its effect on inhibition, destruction (early and mature stages) and in reducing the viability of biofilms formed in respiratory tract infections (Blasi et al., 2016), and indicates its success as an anti-biofilm strategy (Dinicola *et al.*, 2014).

Mixed infections are described to occur in CF and represent a good place for interactions among microorganisms (Layeghifard *et al.*, 2019). For instance, it was supposed that *S. aureus* exerts an effect against *P. aeruginosa* that could prevent the reinfection by this latter and would result in 5 years of increase in the life expectancy of CF patients (Peters et al., 2012).

By the adhesion to mucin assay, we found that *B. cenocepacia* exerts a negative effect on both *S. aureus* or *P. aeruginosa*. In presence of *B. cenocepacia* these pathogens were found be impaired to assess the mucin adhesion sites, as revealed by the exclusion assay. Moreover, even when *B. cenocepacia* was put in the system after these pathogens were already occupying mucin sites, which at first could be considered a disadvantage, *B. cenocepacia has* showed ability to efficiently displace both *S. aureus* and *P. aeruginosa*. *B. cenocepacia* was found to disrupt *S. aureus* biofilms when these bacteria are co-cultivated (Thompson, 2017), which represent another evidence of a negative effect by *B. cenocepacia* on *S. aureus*, that is in congruence with our findings.

We suppose that the inhibition *B. cenocepacia* exerts on *S. aureus* and *P. aeruginosa* could have a clinical relevance in the course of mixed CF pulmonary infections. Usually, CF patients are colonized with *S. aureus* and *Haemophilus influenza* during the first years of life. Then, at younger ages, *P. aeruginosa becomes the most frequently isolated specie, and it is followed by infections with* Bcc bacteria, including *B. cenocepacia* that usually occur in adulthood (Harrison, 2007). Whether we consider that *B. cenocepacia* appears at latter ages in CF patients, and that infections caused by this microorganism are usually fatal, it makes sense to consider that *B. cenocepacia* exerts a deleterious effect on other pathogens, when they compete for a niche in the host.

B. cenocepacia ability to displace and exclude *S. aureus* and *P. aeruginosa* could also trigger effects on the other microorganisms. For example, to compensate the difficulty to gain access to mucin, *S. aureus* and *P. aeruginosa* would counteract using different strategies. Supporting our theory, based on a model of CF pulmonary disease, Bragonzi and colleagues have shown that *P. aeruginosa* biofilms increase biomass and change the architecture during co-infection with *B. cenocepacia* (Bragonzi et al., 2012). This increase biofilm production could represent a good strategy of *P. aeruginosa* to persist and resist *in vivo*. In another work, *Burkdoleria cepacia*, that is close related to *B. cenocepacia*, also senses signs produced by *P. aeruginosa* (Riedel et al., 2001). An unidirectional cross-talk system mediated by N-acylhomoserine-lactone molecules known to regulate biofilms was described during mixed biofilms between *B. cepacia* and *P. aeruginosa* (Riedel et al., 2001). As *B. cenocepacia* and *B. cepacia* are phylogenetically related, it could also exist a similar system in *B. cenocepacia*.

NAC and Hypertonic saline have emerged as two simple, safe, and cost-effectiveness alternative that become to be investigated for CF and had showed many clinical benefits (Tirouvanziam *et al.*, 2006; Conrad *et al.*, 2015) (Conrad *et al.*, 2015). Hipertonic saline, in other hand, has showed to improve the mucociliary clearance and the lung function (Donaldson *et al.*, 2006). They have been showing many benefits in

clinical trials in the last years. The presence of a thick mucus is one of the hallmarks of CF clinics associated to pulmonary exacerbations, and both conditions, individually, seemed to be promising to act in alleviating symptoms, as they are been already used as mucolytic agents.

In conclusion, our data endorsed effectiveness associated to the use of NAC and HS, as these conditions when individually added to the media reduced virulence factor production in *B. cenocepacia*. Inversely, data obtained with NAC associated to NaCl alarm for a possible negative effect on host, which should discourage those who must think in adopt NAC as an alternative treatment in patients with higher NaCl concentrations, as in CF. Biofilm and mucin adhesion in conditions mimicking sub-inhibitory doses of NAC in CF lungs under treatment, showed to increase virulence factors *in vitro*. However, before drawing a definitive conclusion about the effectiveness associated to the use of NAC in CF lungs, these results must be confirmed and expanded. Still, interactions between *B. cenocepacia* and other pathogens had showed that when *B. cenocepacia* is present, this bacteria gets advantage over *S. aureus* and *P. aeruginosa*, inhibiting their adhesion to mucin.

Lastly, although virulence can not be inferred based on *in vitro* tests solely, we can still use them as good tool to investigate conditions related to CF lungs. They can be used on a simple, fast and powerful way to screen potential new strategies of treatments that could affect pathogen virulence. Preliminary data obtained in our study may encourage further investigations directed to test similar conditions on *in vivo* models, as these models are indispensable to infer about a pathogen virulence.

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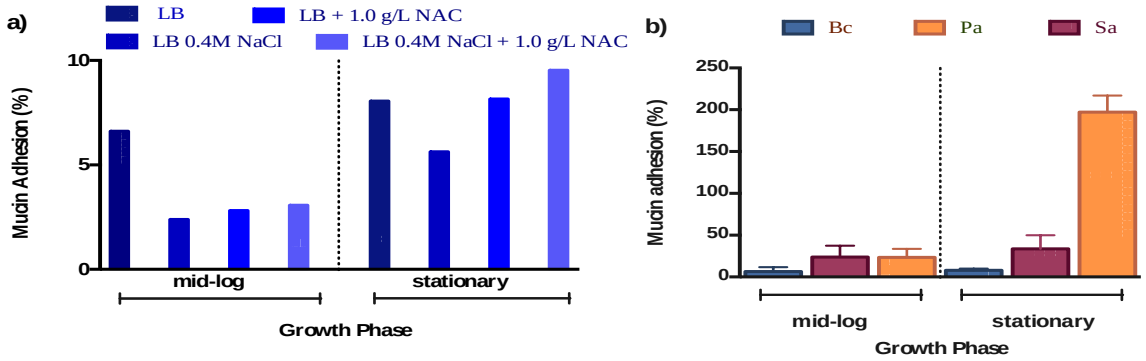

Figure 3: *B. cenocepacia* adhesion values (%) to mucin in mid-logarithmic (mid-log) and stationary phase of growth. Per cent values were calculated from CFU obtained before and after adhesion. In (a), adhesion after growth in LB (control), LB 0.4M NaCL (with or without + NAC at 1g/L) and LB with NAC (1.0 g/L). In Figure (b), *B. cenocepacia* (Bc) adhesion to mucin in comparison with *S. aureus* ATCC25923 (Sa) and *P. aeruginosa* ATCC27853 (Pa). This experiment was performed twice and representative results are shown. Statistical analysis were done with Wilcox Test for two independent samples.

	mid-logarithimic phase			
	S. aureus (ATCC25923)		*P. aeruginosa* (ATCC27853)	
	Exclusion	Displacement	Exclusion	Displacement
B. cenocepacia ET12	79.39 ± 15.68	66.69 ± 2.08	91.92 ± 0.66	14.99 ± 2.55
	stationary phase			
	S. aureus (ATCC25923)		*P. aeruginosa* (ATCC27853)	
	Exclusion	Displacement	Exclusion	Displacement
B. cenocepacia ET12	75.20 ± 29.44	46,63 ± 29,29	96.4 ± 0.29	93.48 ± 0.37

Table 2: Inhibition Assay of *S. aureus* and *P. aeruginosa*.
Relative adhesion of *S. aureus* (ATCC25923) and *P. aeruginosa* (ATCC27853) caused by *B. cenocepacia*. Averages of percentages obtained in two independent experiments + SD were showed.

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