DEPARTAMENT OF
LIFE SCIENCES

NOVA SCHOOL OF
SCIENCE & TECHNOLOGY

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BSc in Cell and Molecular Biology

EVALUATION OF QUATERNARY AMMONIUM SURFACTANTS AS PROPHYLATICS OPTIONS FOR *STREPTOCOCCUS AGALACTIAE* INFECTIONS

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE
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Evaluation of Quaternary Ammonium Surfactants as prophylactic options for *Streptococcus agalactiae* infections

The study presented on this thesis is part of a publication under final preparation and part of the results were presented in the following poster communication:

Carolina Ferreira, Rita Calado, João C. Rodrigues, Otília Vieira, Winchill Vaz, João P. Gomes, Alexandra Nunes. Are quaternary ammonium surfactants a good prophylactic option for *streptococcus agalactiae* vertical transmission? MICROBIOTEC'21. Congress of Microbiology and Biotechnology 2021. Webconference, 23-26 november 2021.

This work was carried out at the department of infectious diseases from the Portuguese National Institute of Health. Part of the work was performed at the "Lysossomes in chronic human pathologies and infection" laboratory, at CEDOC- Chronic Diseases Research Center.This work is part of the project entitled "TITANGEL - Development and evaluation of a surfactant-hydrogel for preventing direct contact transmitted infections", financed by the Fundação para a Ciência e Tecnologia (FCT), which aims to create a safe and effective QAS-containing hydrogel to prevent some bacterial STIs and vertical transmitted *S. agalactiae* infections.

Acknowledgments

Por toda a ajuda e apoio na realização desta dissertação, quero agradecer:

À minha orientadora Dra. Alexandra Nunes, por me ter dado a oportunidade de trabalhar neste projeto, por tudo o que me ensinou, por acreditar em mim e me incentivar sempre a ser melhor e por tudo o resto.

À Dra. Rita Calado, pelo apoio, por tudo o que me ensinou e pelo auxílio sem o qual não teria sido possível.

Ao Dr. João Paulo Gomes, responsável do núcleo de bioinformática do INSA, pela disponibilidade, simpatia a ajuda fundamentais para a realização desta dissertação. Aos restantes membros do núcleo de bioinformática por me terem recebido e ajudado.

Ao Dr. Fernando de Almeida, diretor do Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA), e ao Dr. Jorge Machado, coordenador do departamento de doenças infeciosas, por terem permitido o desenvolvimento desta dissertação no instituto. Em especial, ao Dr. Jorge pela disponibilidade e simpatia durante o meu período no INSA.

À Dra. Maria José Borrego, responsável do laboratório nacional de referência de infeções sexualmente transmissíveis, por permitir a utilização do seu laboratório e pela disponibilidade em partilhar o seu conhecimento para a realização deste trabalho.

A todos os funcionários do INSA com quem me cruzei, em especial ao Dr. João Rodrigues, por todo o auxílio e pela sua disponibilidade e simpatia, fundamentais para a realização do trabalho experimental.

À Dra. Otília Vieira, do Centro de Estudo de Doenças Crónicas da faculdade de Ciências Médicas da Universidade Nova de Lisboa, por ter permitido o desenvolvimento de uma parte deste trabalho experimental no seu laboratório e aos restantes membros do grupo por me terem recebido.

À minha colega Bárbara Alves pelo companheirismo e amizade, sobretudo nas alturas mais complicadas deste trabalho.

À minha família, em especial aos meus pais e irmão, por me terem apoiado, ajudado e motivado em alturas muito difíceis e cansativas em que eu já não acreditava que conseguia.

Aos meus amigos Filipa, Carrazedo, Casinhas, Durval, Almeida, Raquel e Felipe por me aturarem super stressada e por estarem sempre aqui.

À minha amiga Maria, por entender-me, por todo o apoio, motivação e muito mais.

E por último, mas nunca menos importante, a todos os outros que passaram pela minha vida neste último ano e que me apoiaram e ajudaram a ultrapassar os momentos mais difíceis, foram fundamentais para chegar até aqui.

Obrigada!!

ABSTRACT

Streptococcus agalactiae is a leading cause of morbidity and mortality in neonates. Vertical transmission during labor and delivery, is currently prevented by intrapartum antibiotic prophylaxis (IAP). However, resistance to the antibiotics used in IAP has been emerging and increasing. With no vaccine available, it is important to find new alternatives. Previous studies have showed that the cationic surfactant dodecyl-pyridinium bromide (C_{12}PB) is bactericide against *S. agalactiae* at concentrations that do not affect the commensal flora and epithelial cells of the vaginal mucosa. To get a step further in validating C_{12}PB as a good prophylactic compound, this work aimed to evaluate its ability to induce antimicrobial resistance against *S. agalactiae*. Isogenic clones were continuously propagated without and under C_{12}PB sub-inhibitory concentrations. Phenotypic tests coupled with Whole Genome Sequencing of the populations exposed to such conditions were used to identify the emergence and putative mechanisms of resistance. As a proof-of-concept, the same procedure was performed with erythromycin, an antibiotic used in *S. agalactiae* intrapartum prophylaxis. In addition, the toxicity and pro-inflammatory potential of C_{12}PB towards epithelial cells were also evaluated by the MTT assay and ELISA, respectively. Contrarily to erythromycin, where the Minimum Inhibitory Concentration (0.125 µg/ml) doubled after 7 passages and increased up to >2000-times after 56 passages (end point), the appearance of resistance to C_{12}PB was not observed during the assay, which was also confirmed at the genome level. On the other hand, for erythromycin, genome comparison against the original clone revealed multiple mutations on ribosome-associated genes (including the 23S rRNA m5U1939 methyltransferase, ribosomal protein L22 and 23S rRNA) associated with resistance acquisition. Additionally, overall toxicity and inflammation were low. In conclusion, together with the previous results, these findings highlight C_{12}PB as a promising candidate for the prevention of *S. agalactiae* vertical transmitted infections.

Keywords: *Streptococcus agalactiae*, group B *streptococcus*, neonatal infections, intrapartum prophylaxis, antimicrobial resistance, selective pressure assay.

RESUMO

O *Streptococcus agalactiae* é uma das principais causas de morbilidade e mortalidade em recém-nascidos. A transmissão vertical no parto é prevenida pela administração intraparto de antibióticos. No entanto, a resistência aos antibióticos utilizados tem vindo a surgir e aumentar. Com a ausência de uma vacina, é importante encontrar alternativas. Estudos anteriores revelaram que o surfactante catiónico brometo de dodecil-piridínio (C_{12}PB) é bactericida contra *S. agalactiae* em concentrações que não afetam a flora comensal e as células epiteliais da mucosa vaginal. Para estudar o C_{12}PB como um bom composto profilático, este trabalho visou avaliar a sua capacidade de induzir resistência antimicrobiana contra *S. agalactiae*. Para este fim, clones isogénicos foram continuamente propagados na presença e ausência de concentrações sub-inibitórias de C_{12}PB. Para identificar a emergência e os mecanismos putativos de resistência, foram utilizados testes fenotípicos e sequenciação do genoma inteiro das populações expostas a estas condições. Como prova de conceito, o mesmo procedimento foi realizado com a eritromicina, um antibiótico utilizado na profilaxia intraparto de *S. agalactiae*. A toxicidade e o potencial pró-inflamatório do C_{12}PB para células epiteliais foram também avaliados através do ensaio do MTT e ELISA, respetivamente. Contrariamente à eritromicina, onde a Concentração Mínima Inibitória (0,125 µg/ml) duplicou após 7 passagens e aumentou até >2000 vezes após 56 passagens (final do ensaio), o aparecimento da resistência ao C_{12}PB não foi observado, o que foi confirmado a nível genómico. Por outro lado, para a eritromicina, a comparação de genomas contra o clone original revelou mutações no rRNA 23S, metiltransferase m5U1939 do rRNA 23S e proteína ribossómica L22, alvos associados à aquisição de resistência. Além disso, os níveis de toxicidade e inflamação foram baixos na generalidade. Concluindo, juntamente com os resultados anteriores, estas descobertas destacam o C_{12}PB como uma opção promissora para a prevenção de infeções neonatais causadas *por S. agalactiae.*

Palavras chave: *Streptococcus agalactiae*, *Streptococcus* hemolítico do grupo B, infeções neonatais, profilaxia intraparto, resistência antimicrobiana, ensaio de pressão seletiva.

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SYMBOLS, ABBREVIATIONS AND ACRONYMS

α	Alfa
β	Beta
γ	Gama
AAP	American Academy of Pediatrics
ACOG	American College of Obstetricians and Gynecologists
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
Bp	Base-pairs
C_{12}PB	Dodecyl-pyridinium bromide
CC	Clonal complex
CDC	Center for Disease Control
CMC	Critical micelle concentration
cMLSB	Constitutive macrolide-lincosamide-streptogramin B resistance
CPS	Capsular polysaccharide
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EOD	Early-onset disease
ERY	Erythromycin
EUCAST	European Committee on Antimicrobial Susceptibility Testing
erm	Erythromycin ribosome methylase
FBS-HI	Heat inactivated fetal bovine serum
GBS	Group B Streptococcus
h	Hours
IAP	Intrapartum antibiotic prophylaxis
ICEs	Integrative and conjugative elements
$iMLS_B$	induced macrolide-lincosamide-streptogramin B resistance
LOD	Late-onset disease
Mef	Macrolide efflux
MIC	Minimum inhibitory concentration
Min	Minutes
MLS_B	Macrolide-lincosamide-streptogramin B resistance
MLST	Multilocus Sequence Typing
o/n	Overnight
PBS	Phosphate buffered saline
QAS	Quaternary Ammonium Surfactants

RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
rpm	Revolutions *per* minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
Snp	Single nucleotide polimorphism
ST	Sequence type
STI	Sexually transmitted infections
USA	United States of America
UK	United Kingdom
v/v	Volume *per* volume
WGS	Whole Genome Sequencing

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1

INTRODUCTION

1.1 Streptococcus agalactiae - general overview

Streptococcus agalactiae (*S. agalactiae*) is a gram-positive bacterium belonging to the Firmicutes phylum; *S. agalactiae* is a coccus, with tendency to form short or long chains, non-motile, beta-hemolytic, catalase-negative, and facultative anaerobe[1].

During the early 1930's, *S. agalactiae* was first identified as a veterinary pathogen after it was isolated from milk and cows with bovine mastitis, and it was classified as group B *Streptococcus* (GBS) of the Rebecca Lancefield classification of streptococci[2]. In the late 1930's, human pathogenicity was first reported, as cases of fatal postpartum infection[3,4] and in the 1970's *S. agalactiae* emerged as a leading cause of neonatal illness and mortality[5,6]. Currently, the situation improved due to the medical advances and the implementation of prevention measures, but *S. agalactiae* remains an important cause of disease burden worldwide and a global health concern[7].

1.2 Colonization, infection, and disease

S. agalactiae commonly colonizes the gastrointestinal and genitourinary tracts, being carried by up to 33% of adults, usually asymptomatically[8,9]. Transmission is thought to occur via fecal-oral, sexual, or through vertical transmission[10].

Despite *S. agalactiae* colonization is, in general, harmless, it represents a serious concern in pregnant women[11]. Indeed, during pregnancy and postpartum, *S. agalactiae* can lead to a range of invasive bacterial diseases in carrier women, such as meningitis, osteomyelitis and endocarditis and noninvasive diseases, including bacteriuria, fasciitis, endometritis, and wound infections associated with episiotomies or cesareans[11,12]. Besides the risk for pregnant women, *S. agalactiae* colonization may also have serious consequences in newborns as ~30-70% of carrier mothers deliver colonized infants[13]. Infection

may result from the ascending spread of *S. agalactiae* from the vagina into to the uterus and/or placenta, or at birth, during passage through the birth canal[14]. Neonatal invasive *S. agalactiae* diseases, which develop within the first 7 days of life, are designated early-onset disease (EOD) and usually manifest as pneumonia and sepsis[9,13]. Mortality rates for EOD vary worldwide. In well-resourced countries, the case fatality risk is about 5%, while in developing regions (*e.g.* Africa) the rate is much higher, up to 27%[9]. On the other hand, late-onset disease (LOD) develops in infants aged >1 week and are normally associated with meningitis, where up to 50% of the survivors develop chronic neurologic sequelae[13]. *S. agalactiae* infection has also been associated with increased risk of neonatal encephalopathy and associated mortality[15]. In addition, ascending *S. agalactiae* infection from the vagina into the uterus and gestational tissues may cause chorioamnionitis or inflammation of the placenta, which can result in stillbirths and preterm births[12]. In fact, prematurity is one of the main causes of neonatal death, accounting for 44% of all deaths under 5 years of age and most of these preterm births are caused by microbial infection with *S. agalactiae* being responsible for about 10%[12,16,17]. In fact, according with the World Health Organization (WHO), *S. agalactiae* was linked to more than half a million preterm births and at least 46,000 stillbirths in 2020[7]. Overall, mortality rates for neonatal *S. agalactiae* disease are 1-8 % in full-term infants and 5-20% in preterm infants[9].

Although *S. agalactiae* infection is particularly recognized as a problem in pregnant women and newborns, it is not restricted to these groups. According to the Center for Disease Control (CDC), *S. agalactiae* infection also affects non-pregnant adults (10 cases/100000) and people with ≥65 years of age (25 cases/100000)[18]. *S. agalactiae* disease in non-pregnant adults includes pneumonia, arthritis, endocarditis and skin, soft tissue, and bone infections, mainly in elderly and immunocompromised patients[9,18]. The mortality rate of serious GBS infections in non-pregnant adults is about 1 in 20, according to CDC[18].

1.3 *S. agalactiae* classification and epidemiology

S. agalactiae serotype classification is based on a major virulence factor named Capsular polysaccharide (CPS), encoded by the *cps* gene cluster[13,19]. Currently, there are ten *S. agalactiae* capsular serotypes (Ia, Ib, II – IX) based on the identification of specific CPS antigens[19]. *S. agalactiae* serotype identification can be performed by numerous methods, including the classic Lancefield precipitation test (LP test), latex agglutination, enzyme immunoassay, capillary precipitation and even by identification of its capsular genotype, by PCR and sequencing of the *cps* gene clusters[20-23].

Globally, serotypes Ia, II, III and V account for 98% of *S. agalactiae* colonizing pregnant women, but serotype distribution and prevalence varies across the world[24]. For instance, serotypes Ia, II, III, and V are the most common in Europe and in the United States of America (USA)[13,25,26], whereas serotype

V is the most prevalent in Africa[24,27] and serotypes VI and VIII in Japan[28,29]. In Portugal, accordingly with the available data, serotypes III and V are the most prevalent[25,30,31].

In the last decade, Multilocus Sequence Typing (MLST), a more detailed classification system that groups strains based on nucleotide diversity of seven housekeeping loci (*adhP*, *pheS*, *atr*. *glnA*, *sdhA*, *glcK* and *tkt*) into sequence types (ST) and in clonal complexes (CC), has been used to identify genetic lineages[32]. The majority of the human *S. agalactiae* isolates can be grouped into six CCs which are CC1, CC10, CC17, CC19, CC23, and CC26[33,34]. CC1, CC17, CC19 and CC23 are the dominant clones in pregnant women[13].

GBS disease outcome (*e.g*. carriage *versus* invasive disease) is typically associated with *S. agalactiae* CC and serotype. One example is the *S. agalactiae* III/CC17 hypervirulent clone, which accounts for most cases of late-onset meningitis in newborns, whereas CC1, CC19 and CC13 are largely associated with asymptomatic colonization[13]. Additionally, a Portuguese study on colonizing and invasive *S. agalactiae* isolates[31], revealed that serotypes III and V were the most prevalent among the 269 colonization isolates (44%), while serotypes Ia and III represented 69% (44/64) of the invasive isolates. In particular, serotype Ia was mostly responsible for EOD, while serotype III was associated with LOD[31].

One of the largest studies performed, comprising data from 299 924 pregnant women in 85 countries, estimated the *S. agalactiae* colonization rates (fig 1)[24]. Worldwide, the overall prevalence of *S. agalactiae* colonization in pregnant women is about 18%, varying from 11% in Eastern Asia to 35% in the Caribbean[24]. In Europe, the colonization prevalence among pregnant women varies between 17.6 % in southern Europe to 23 % in western Europe[24]. In Portugal, the knowledge of *S. agalactiae* epidemiology is limited. Florindo *et al.*[25] reported a colonization rate of 6.2% in samples recovered from women of reproductive age during the period 2005-2007 in the region of Lisbon[25].

Importantly, the new report from the WHO[7] (addressed in November 2021) reveals that the global burden of *S. agalactiae* is higher than previously recognized, with 390 000 infant cases, 91 000 newborn deaths and leading to neurological sequalae in 40 000 infants in 2020[7]. The global rate of S. agalactiae maternal colonization was about 15% (totaling nearly 20 million women worldwide)[7].

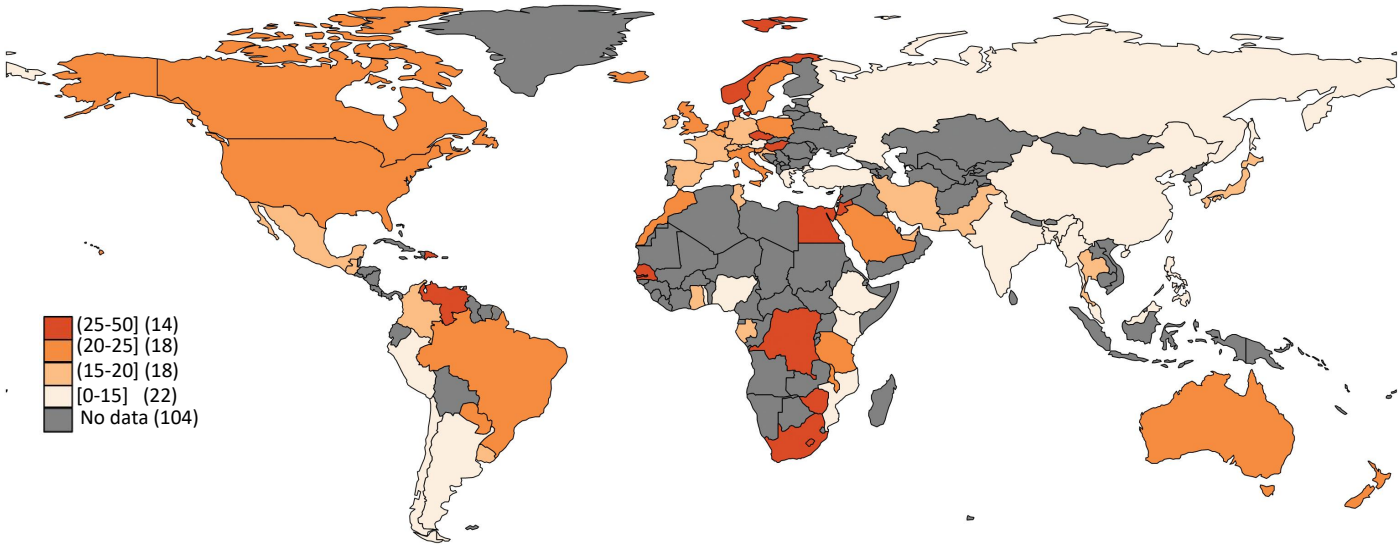

Figure 1.1. Prevalence of *S. agalactiae* maternal colonization by country. Data is adjusted for sampling site and laboratory culture method. (Retrieved from "Maternal Colonization With Group B *Streptococcus* and Serotype Distribution Worldwide: Systematic Review and Meta-analyses" [24]).

1.4 Virulence factors and mechanisms

S. agalactiae has multiple virulence factor and mechanisms. The CPS is one of the major virulence factors and has a central role in immune evasion through a diversity of mechanisms including interference with complement-dependent defense mechanisms, inhibition of phagocytosis by the neutrophils and facilitation of bacterial survival inside dendritic cells[12,13,35]. The adhesins that mediate the interaction with host cells are important virulence factors and include the group B streptococcal C5a peptidase (encoded by the *scpB* gene), the hypervirulent adhesin HvgA and the GBS immunogenic bacterial adhesin BibA. Bacterial biofilms are also a known mechanism of virulence in *S. agalactiae*[12,13].

In other *Streptococcus* species, such as *Streptococcus pneumoniae*, a phenomenon referred as serotype switching or capsular switching, consisting of "a change of serotype of a single clone by alteration or exchange of its cps locus" is common and well described as a virulence mechanism[36,37]. In *S. agalactiae*, although rarer, this occurrence has also been identified[37,38].

1.5 Prevention and treatment

Since the most significant problem concerning *S. agalactiae* is the neonatal disease, prevention measures are established to decrease the burden of these diseases. In 1996, the American College of Obstetricians and Gynecologists (ACOG), the American Academy of Pediatrics (AAP) and the CDC issued guidelines to prevent perinatal *S. agalactiae* disease[6]. These guidelines recommended intrapartum antibiotic prophylaxis (IAP) for the women with an increased risk of delivering *S. agalactiae* colonized infants, to prevent vertical transmission during labor[6]. The guidelines were followed and/or adapted by many countries[8,38,39]. According to WHO, in 2017 sixty countries had policy for the use of antibiotics in pregnancy to prevent *S. agalactiae* infections[8]. Women are routinely screened for *S. agalactiae* colonization between 35 to 37 weeks of gestation, by a rectovaginal swab. If the result is positive, intrapartum antibiotics (IA) are then administered during labor[6,8,38,39]. The administration of IAs is also performed in other situations such as in prior history of *S. agalactiae* perinatal infection and/or when women suffer prolonged rupture of membranes[6,38,39].

Penicillin is the antibiotic of choice for the prophylaxis and treatment of GBS infections. However, in cases of severe penicillin allergy or risk for anaphylactic reactions, other antibiotics such as erythromycin, clindamycin, vancomycin, and cefazolin can be used[9,18,40]. However, strains resistant to these antibiotics have been emerging, and the prevalence of antimicrobial resistance (AMR) among *S. agalactiae* strains is increasing[40].

Apart from the antibiotic resistance, the current strategy for *S. agalactiae* prophylaxis has other issues. *S. agalactiae* colonization is intermittent and highly variable during pregnancy, thus the initial screening at 35-37 weeks of gestation may not reflect a real infection scenario at delivery[41]. This can lead to a missed opportunity to administrate IAPs or, in the opposite scenario, to the unnecessary use of antibiotics, which ultimately contributes to the increase of AMR[41]. In addition, unlike the developed countries, screening and IAP are in general not implemented in low- and middle-income countries (where the largest burden of GBS is seen, with sub-Saharan Africa accounting for half of the global burden[42]) due to limited resources and infra-structures[27,42].

The WHO and the Product Development for Vaccines Advisory Committee (PDVAC) defined the development of a GBS vaccine and its use in low- and middle-income countries as a priority in 2015[42].

Efforts have been made to develop a vaccine for *S. agalactiae*, and some vaccine candidates have been or are currently being studied in clinical trials[42-47]. However, most vaccines candidates are based on the CPS antigen, and therefore, can only prevent infection by specific serotypes. Hence, the increasing diversity of *S. agalactiae* serotypes together with its geographically diversity and the serotype switching, have made this task difficult[13]. For instance, even trivalent (serotypes Ia, Ib and III) and

pentavalent (serotypes Ia, Ib, II, III and V) vaccine candidates did not include serotype IV, as this serotype was not one of the major concerns[44,46,47]. However, serotype IV has recently emerged as an invasive clone[13,30]. In support of this, Florindo *et al.*[30] described an increase in the prevalence of serotype IV, among isolates from reproductive age women in the region of Lisbon, from 1% in 2006 to 20% in 2012. An additional concern with the development of a vaccine is that serotypes that are not typically associated with invasive disease, may become invasive, a phenomenon that had been observed with vaccines against *Streptococcus pneumoniae* [12,48].

Numerous vaccines based in conserved surface proteins, rather than the CPS, have been considered and studied[49-52]. The WHO recently estimated that *S. agalactiae* vaccination could prevent over 31 000 newborn deaths and 21 000 cases of neurodevelopment impairment, however to this date there is no *S. agalactiae* vaccine available[42.] Hence, there is an urgent need for novel prophylactic options for *S. agalactiae* infections[42].

The development of new approaches for the prophylaxis or treatment of any condition can include the formulation of a new drug or the use of existing compounds in a new way, using drug delivery systems. Concerning the administration route, *in situ* applications present advantages in comparison to oral or intravenous (currently used in IAP) routes[53,54]. The local delivery of the drug not only allows to avoid its systemic effects but also minimizes the development of antimicrobial resistance[53,54]. In fact, the use of polymeric gel formulations was already studied as vaginal *in situ* delivery system[53].

1.6 Antibiotic resistance

AMR is recognized as a major global health problem. This issue has led to extreme measures such as the use of multiple antibiotics for the treatment of some infections and the discontinuation in the use of several antibiotics, leading to the need of third-line antibiotics for some bacteria[55]. Indeed, in some countries, including Portugal, due to the dramatic increase on *S. agalactiae* AMR, clindamycin and erythromycin are no longer recommended as second-line antibiotics for IAP[38,39].

Erythromycin and clindamycin are chemically different antibiotics, belonging to the Macrolides and lincosamides group, respectively[40]. However, their mode of action is similar, preventing protein synthesis by binding to the bacterial large ribosomal subunit, specifically to the 23S subunit for the macrolides[40,56]. For that reason, resistance to one class of antibiotics often confers cross-resistance to the other, which is stated as the MLSB phenotype[40]. This phenotype can be constitutive (c-MLSB) or inducible (i-MLSB)[56].

Resistance can be conferred through different mechanism including: i) ribosomal modifications, ii) efflux pumps and iii) drug inactivation[56]. A common mechanism of resistance to macrolides in *S.*

agalactiae is through macrolide efflux (Mef) pumps, encoded by the *mefA* and *mefE* genes[40,56]. This originates the M phenotype, conferring resistance to macrolides but not to lincosamides[56]. Differently, the MLSB phenotype is commonly conferred by ribosomal modification[50,56]. These modifications mostly correspond to posttranscriptional modifications at the 23S rRNA, by methylases coded by the *erm* (erythromycin ribosome methylase) genes[40,56]. Another type of ribosomal modifications that confer erythromycin resistance is the presence of mutations on ribosomal proteins L4 and L22 that cause conformational alterations within the 23S rRNA[56,57].

High prevalence of *S. agalactiae* isolates exhibiting erythromycin resistance have been described in several countries such as China (74.1% in both colonizing and invasive isolates), USA (54.8% and 44.8% in invasive isolates for adults and infants, respectively) and Italy (43.75% in maternal colonization isolates)[58-62]. The same has been reported for clindamycin, with high resistance rates being described in Taiwan (65.9% in invasive isolates from infants), Algeria (43.2% in invasive disease isolates) and Portugal (34% in invasive isolates from non-pregnant adults)[63-65].

In Portugal, a study of isolates recovered from women of reproductive age during the period 2005-2007 in the region of Lisbon[26], reported clindamycin and erythromycin resistance rates of 10% and 19% respectively. More recently, a similar study with isolates recovered during the period 2005-2012 in the region of Lisbon[30], reported that the prevalence of erythromycin resistance ranged from 14% in 2006 to 23% in 2011 while clindamycin resistance rates ranged from 6% in 2009 to 18% in 2012.

Even for penicillin, known for being universally effective against *S. agalactiae*, resistance has already been reported. The first description of penicillin resistance was in 2008 in Japan in isolates recovered from non-pregnant adults, conferred by amino acid substitutions near the active site of the penicillin binding protein PBP2x[66]. Since then, resistance has also been reported in Canada, Korea, and in the USA[67-71]. Additionally, a study in Japan[72] reported an increase in penicillin resistant *S. agalactiae*, with resistance rates ranging from 2.3% in 2005-2006 to 14.7% in 2012-2013. Moreover, a recent review of *S. agalactiae* maternal colonization strains in Africa[73] reported penicillin as the second antibiotic with the highest pooled proportion of antimicrobial resistance (33,6%).

Importantly, *S. agalactiae* strains resistant to multiple antibiotics, including penicillin, have been emerging [72,74-76]. Another important matter is that resistance to vancomycin, mediated by the acquisition of a resistance gene, *vanG*, has been observed in the USA, which is really concerning since vancomycin is a last-resource antibiotic[77,78].

1.7 Quaternary Ammonium Surfactants (QAS)

Surface Active Agents (surfactants) are substances that reduces a liquid surface tension when added to it, or lower the interfacial tension between two liquids, a solid and a liquid or a gas and a liquid. Surfactants are usually organic compounds that are amphiphilic, consisting of a hydrophilic head and a hydrophobic tail[79,80]. Surfactants may act as detergents, emulsifiers, wetting agents, dispersants, and foaming agents and are therefore broadly used for several purposes such as personal care products (skin cleansers, shampoo...), cosmetics, pesticides, pharmaceuticals, in biotechnology and in the oil recovery industry[81-84].

An important propriety of these compounds is the formation of micelles (aggregates of surfactant molecules in a colloidal solution). In aqueous solutions, surfactant molecules typically form aggregates with the hydrophobic tails sequestered in the micelle centre, surrounded by the hydrophilic heads facing the aqueous solvent. The concentration of surfactant at which micelle formation starts to occur is the critical micelle concentration (CMC). This concentration is an important parameter since surfactants properties are altered by the formation of micelles[85].

There are different types of surfactants, classified based on their dissociation in water: i) Anionic surfactants dissociate in an amphiphilic anion and a cation; ii) cationic surfactants dissociate in an amphiphilic cation and an anion; iii) nonionic surfactants do not ionize in aqueous solution; and iv) zwitterionic surfactants exhibit both anionic and cationic dissociations. The groups of surfactants possess different properties and are therefore used for different purposes[78,79]. For instance, cationic surfactants are the most effective bactericides and germicides and have long been known for having a broad-spectrum microbicidal activity[86]. For that reason, these surfactants are generally employed as antiseptics and disinfectants[79,80,86]. Due to the well-known broad-spectrum activity, some studies in the STI field were performed using surfactants as microbicides[87-90]. Although some of these studies reached Phase III of clinical trials surfactants disappointed by inducing toxicity and damage into the vaginal mucosa, which dropped away the pharmaceutical interest[91-93].

QAS, also known as quaternary ammonium salts and/or quaternary ammonium compounds, are the most representative sub-family of cationic surfactants. This group is distinguished as the most useful disinfectants and antiseptics, being widely used in domestic, industrial, and clinical applications[79,94,95]. QAS are amphiphilic molecules with a positively charged quaternary ammonium polar head group, having one or two apolar chains, usually n-alkyl, attached to it (fig 1.2)[80]. These compounds are chemically stable, possess non-demanding storage requirements and a low price. Along with their broad-spectrum antimicrobial activity, these characteristics makes QAS attractive candidates for prophylaxis of bacterial infections in low-resourced regions[96].

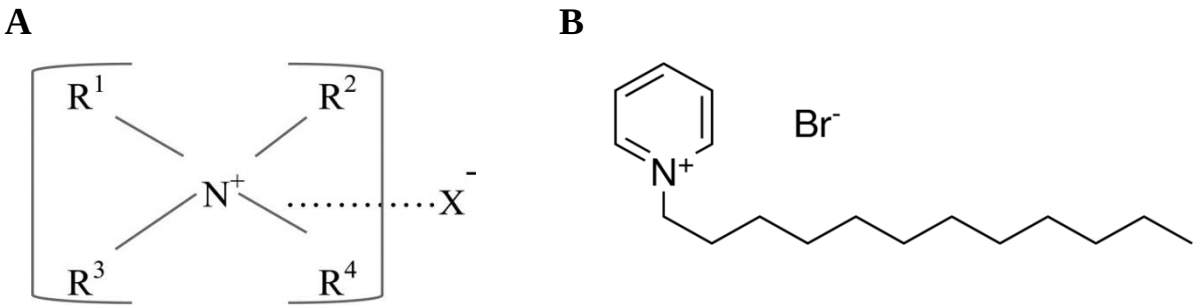

Figure 1.2. QAS basic structure (A) and Docecyl-pirydinium bromide chemical structure (B). The X represents the anion, usually a halide (e.g chlorine or bromine). R^1-R^4 are normally aryl or alkyl groups and QAS are classified based on the nature of these groups (length and branching of the carbon chain, number of nitrogen atoms, presence of aromatic groups…). (Adapted from "Quaternary Ammonium Biocides: Efficacy in Application"[79] and from https://www.sigmaaldrich.com/PT/en/product/aldrich/s519626)

Previous studies have already showed that cationic monoalkyl QAS, when used at concentrations lower than the CMC, may work as selective bactericides, without affecting mammalian cells *in vitro*[93]. Moreover, QAS toxicity mechanisms towards bacterial and mammalian cells were found to be different[96,97]. While toxicity to bacterial cells involves impairment of bacterial energetics and cell division at low concentrations as well as permeabilization of the cell membrane and electron transport inhibition at slightly higher concentrations[96]; toxicity to mammalian cells occurs at higher concentrations and is mainly due to mitochondrial dysfunction[97]. Furthermore, QAS with short n-alkyl groups (C_{10}-C_{12}) were found to be more efficient and selective microbicides when compared to those with 14-16 carbons[98]. Moreover, *S. agalactiae* was much more susceptible to short (C12) n-alkyl QAS toxic effects than the commensal vaginal flora, namely the *Lactobacillus* species[98].

1.8 Scope of the thesis

Recently, from a set of three C(12) n-alyl-QAS analogues tested [dodecyl-piridinium bromide (C_{12}PB), dodecyltrimethylammonium bromide (C_{12}TAB) and dodecyl-N-benzyl-N,N-dimethylammonium bromide (C_{12}BZK)], the analogue with the pyridinium bromide (PB) polar head, C_{12}PB, was found to be the most effective microbicide[97]. In order to take a further step in validating C_{12}PB as an alternative prophylactic option for GBS infections, the present work had two main goals:

i) to evaluate C_{12}PB toxicity and pro-inflammatory profile towards *in vitro* models of the surfactant's target tissue, the vaginal epithelial mucosa;

ii) to evaluate C_{12}PB ability to induce antimicrobial resistance in *S. agalactiae* as well as its putative mechanism of action.

Bacterial resistance towards QAS commonly used in food and cosmetic industries has been described, mostly in Staphylococcus aureus[94,97]. The mechanisms of resistance to QAS are described for some bacteria but seems to exist no data concerning *S. agalactiae* or C_{12}PB[94,97]. By evaluating the development of resistance in a QAS-susceptible *S. agalactiae* clinical isolate, the present study pretends to contribute to fill this gap. Indeed, the characterization of the putative antimicrobial resistance genetic mechanisms to C_{12}PB may allow the identification of genetic markers responsible for different levels of resistance, which may be useful for rapid screening studies in clinical settings, by using PCR or molecular arrays targeting the identified genetic features. Moreover, it may shed some lights regarding which molecular pathways are likely controlling the phenotypic expression of putative C_{12}PB resistance, allowing to understand if this compound trigger the same type of resistance mechanisms as for the commonly used antibiotics.

2

MATERIAL AND METHODS

2.1 Cell culture and treatment

To evaluate C_{12}PB *in vitro* toxicity and pro-inflammatory potential towards human epithelial cells, human cervical epithelial cell line HeLa (ATCC® CCL-2™) and human intestinal columnar epithelial cell line Caco-2 (ATCC® HTB-37™) were selected. Both cell lines mimic the different types of cells existent on the human cervicovaginal mucosa. For the viability assays, both cells were seeded in 96-microwell plates (Thermo Fisher Scientific, Waltham, USA) at an initial density of approximately 20000 cell/well for HeLa and 32000 cell/well for Caco-2, in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX™ supplemented with 10% heat inactivated fetal Bovine serum (FBS HI), 100 U/ml penicillin and 100 μg/ml streptomycin. For Caco-2, medium was also supplemented with 1% of sodium pyruvate and 1mM of non-essential amino acids. The plates were incubated at 37 ºC in 5% CO_2. For HeLa cells, a confluence of 80-100% was obtained after 24h of incubation, whereas Caco-2 cells were maintained in culture for ten days, in order to get confluent and fully polarized.

For the cell viability assays, C_{12}PB (Sigma-Aldrich, USA) stock solutions were prepared in reduced serum medium (Opti-MEM™), as submultiples of the CMC, ranging from $6.5x10^{-4}$ mM to 3.9 mM. Cells were then incubated with increasing concentrations of C_{12}PB for 20, 60, 180 and 360 minutes, at 37 ºC in 5% CO_2. Opti-MEM without surfactant was used as a control. After each incubation, the medium was collected and replaced with complete fresh DMEM without phenol red, supplemented as described above for DMEM GlutaMAX™. The plates were then incubated for more 24 h at 37 ºC in 5% CO_2 and cell viability was accessed by the MTT assay. During the procedure media were collected from each well, centrifuged for 6 minutes at 1200 rpm and stored at -80 ºC for further use. All incubations were done in triplicate. Reagents used in cell culture were all from Gibco™ (Thermo Fisher Scientific, Waltham, USA).

2.2 Cell viability assessment by MTT assay

To assess cell viability after C_{12}PB exposure, cells were incubated with MTT reagent [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (Sigma-Aldrich, St. Louis, USA) diluted in PBS supplemented with 0,9 mM Ca^{2+} and 0,49 mM Mg^{2+}], with a final concentration of 1 mg/mL, for 2 h at 37 ºC in 5% CO_2, protected from the light. To dissolve formazan crystals, formed during the reaction, 100 µl of solubilization solution (16% SDS in 50% DMSO) were added to each well and the plates were kept overnight at room temperature (RT), protected from the light. The samples were quantified colorimetrically at 570 nm (background wavelength correction at 620 nm) on a SpectraMax® i3x spectrophotometer (Molecular Devices, San José, USA). Background absorbance (culture medium plus MTT without cells) was subtracted from the absorbance of each sample and the results are shown as a percentage of the control.

2.3 Characterization of C_{12}PB in vitro inflammatory potential by Enzyme-Linked Immunosorbent Assay (ELISA)

After analyzing the results of the MTT assay, a range of subtoxic concentrations of C_{12}PB were selected for each cell line (25 µm, 7.8 µm and 3.9 µm for HeLa cells and 39 µm, 25 µm and 3.9 µm for Caco-2 cells) and the respective cell supernatants (collected in the previous task) were analyzed for the presence of pro-inflammatory human cytokines and chemokines commonly used as biomarkers of inflammation in vaginal microbicide studies[99-101]. The procedure was performed with the commercial uncoated ELISA kits (Invitrogen, Thermo Fisher Scientific, USA), according to the manufacturer's instructions with minor alterations. Briefly, Nunc™ Maxisorp™ 96-well plates (Thermo Fisher Scientific, Waltham, USA), were coated with anti- IL-8, anti-IL-6, anti-TNF-a, anti-IFN-g, anti-IL-1ß, anti-MCP-1/CCL2, anti-MIP-1/CCL3 and anti-IL-10 (anti-inflammatory) antibodies and incubated overnight at 4°C. A standard curve was performed according to the manufacturer's instructions.

The plates were washed with PBS 1x, 0.05% Tween and non-specific binding was blocked by incubating with 100 µl of ELISASPOT diluent for 1 h, at RT. Plates were washed again and 50 µl of each sample were added to each well, followed by the addition of the same volume of detection antibody and incubation for 1 h at RT. The plates were incubated with 50 µl of the enzyme Streptavidin-HRP for 30 min at RT, washed and incubated with 50 µl of TMB solution for 15 min at RT. Finally, stop solution was added and the absorbances at 450 nm were read on a SpectraMax® i3x spectrophotometer (Molecular Devices, San José, USA), using a reference filter at 570 nm. For each cytokine, the assay was

performed in triplicate (n=3). Controls of the assay were obtained by exposing the cells to several immune stimulus (table 1) or medium only.

Table 1. Positive controls used in ELISA

Cytokine/ chemokine	Positive controls	
	HeLa cells	**Caco-2 cells**
IL-6	IFN-γ+IL-1β+TNF-α and IL-1β	IFN-γ+ IL-1β+TNF-α
IL-8	IL-1β	IFN-γ+ IL-1β+TNF-α
IL-1β	TNF-α	IFN-γ+ IL-1β+TNF-α
TNF-α	IFN-γ	IFN-γ
MCP-1/CCL2	IFN-γ+ IL-1β+TNF-α	IFN-γ+ IL-1β+TNF-α
MIP-1/CCL3	IL-1β	IFN-γ+ IL-1β+TNF-α
IL-10	IL-10	IFN-γ+ IL-1β+TNF-α

2.4 Evaluation of AMR Induction

Publications with *S. agalactiae* selective pressure assays are scarce, so the data concerning this issue is limited. Therefore, an "evolutionary *in vitro*" approach that has been proven to accurately mimic natural evolution in several pathogens[102], including the characterization of *Staphylococcus aureus* and *Pseudomonas aeruginosa* resistance mechanisms to last-line antibiotics[103,104], was applied for C_{12}PB. For comparative purposes, AMR evaluation was performed, in parallel, for an antibiotic that can be used in *S. agalactiae* IAP and it is known to induce antimicrobial resistance: Erythromycin (ERY).

In order to optimize the conditions and methodologies for the assay, numerous experiments had to be performed. These experiments are briefly described in appendix A.

The *S. agalactiae* clinical isolate (designated as CL-1) used in this study belongs to the stock culture collection of the STI Reference Laboratory from the Portuguese National Institute of Health. CL-1 previous characterization included the following characteristics: Serotype V; ST19/ CC19 and phenotypic resistance to tetracycline.

2.4.1 Minimum Inhibitory Concentration (MIC) determination

The MICs for C_{12}PB and ERY (Sigma-Aldrich, USA) were determined by the broth microdilution method, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[105]. Briefly, twofold serial dilutions of C_{12}PB or ERY were prepared in Todd Hewitt Broth (Thermo Fisher Scientific, Waltham, USA) to achieve concentrations ranging from 0.390625 to 200 µM for the surfactant and from 0.00390625 to 2 µg/ml for the antibiotic. An inoculum of 1.5×10^8 CFU/ml

was prepared by direct colony saline–phosphate-buffered saline (PBS) suspension, using colonies from overnight 5% sheep blood agar plates. A 1:20 dilution of the bacterial suspension was added to each well to obtain a final concentration of 5×10^5 CFU/ml. For each plate, controls containing Todd Hewitt broth with and without inoculum were used. The plates were then incubated at 37 ºC in 5% CO_2. After 20-22 hours, growth was analyzed by direct observation of the plates and by measuring the optical densities (OD) at 620 nm using the microplate reader (Quilaban).

For ERY, MICs were also determined by ETEST® (bioMérieux) using Todd agar (for comparative purposes with the microdilution method) and Columbia agar + 5% sheep blood (as recommended by EUCAST). Basically, ETEST® strips were placed onto plates inoculated with a sterile cotton swab previously dipped into the bacterial 0.5 McFarland suspension. Plates were then incubated at 37 ºC with 5% CO_2 for 20-22 hours.

MICs were determined as the lowest C_{12}PB or ERY concentration at which no visible growth was observed. For all methodologies, MICs were determined in three independent assays.

2.4.2 Growth curves

Bacterial growth curves were performed in the presence and absence of C_{12}PB and ERY. 24-well plates were prepared with negative controls (Todd Hewitt Broth), positive controls (broth with bacteria) and broth with 1% v/v of C_{12}PB or erythromycin with different concentrations (MIC, $\frac{1}{2}$ MIC, $\frac{1}{4}$ MIC and $\frac{1}{8}$ MIC). The inoculum was prepared as previously described in 2.4.1, to obtain a final concentration of 5×10^5 CFU/ml *per* well. Plates were incubated at 37 ºC in 5% CO_2. Growth was monitored by measuring the ODs at 600 nm, using a GeneQuant Pro spectrophotometer (Amersham Biosciences, Amersham, UK) at the following timepoints: 4h, 6h, 7h, 8h, 9h, 10h, 11h, 12h, 13h, 14h, 15h, 18h, 20h. The mean values, of two replicates, for each timepoint were calculated.

2.4.3 *In vitro* selective pressure assay

Isogenic clones of the *S. agalactiae* CL-1 ($5x10^5$ CFU/ml) were continuously sub-cultured on a 24-well plate, at the exponential growth phase, in Todd-Hewitt medium under the following conditions (fig. 2.1): i) with a constant C_{12}PB/ ERY $\frac{1}{4}$ MIC (1% v/v), eight replicates; ii) without C_{12}PB/ ERY (positive controls), four replicates, to discard mutations that may emerge due to laboratory passages. Four negative controls were also included, two containing fresh Todd-Hewitt broth and two passed from the previous plate to check for any potential slow development contamination on the plate

After incubation for 12 hours at 37 ºC in 5% CO_2, the ODs at 600 nm were measured on GeneQuant Pro spectrophotometer (Amersham Biosciences) to monitor the bacterial growth and to

determine the right amount of culture to transfer to a new plate, without creating a bottleneck (fig 2.1). This procedure was repeated every 12 hours for 29 days. Whenever any MIC alteration was observed, the sub-MIC used in the assay ($\frac{1}{4}$ MIC) was rectified.

The content of all wells except negative controls was collected once a day and stored at ⁻ 20 ºC for DNA extraction and WGS, as well as every three days at ⁻ 80 ºC in 25% glycerol to preserve the cultures.

2.4.3.1 Phenotypic tests

To follow MIC alterations during the continuous passages, phenotypic tests were performed every six passages. For that purpose, 35 µl of bacterial suspension from each well were added to a new 24-well plate prepared with Todd Hewitt broth containing C_{12}PB or ERY at concentrations corresponding to the MIC and 2×MIC (1% v/v) (see fig 2.1). The plates were then incubated at 37 ºC in 5% CO_2 and after 12 hours the presence or absence of growth in each well was observed and registered. In accordance with the MIC alterations observed in each passage tested, other multiples of the MIC were included in the test (up to 16 times-MIC). For ERY, when the MIC increases were no longer possible/practical to follow by broth dilution, this method was replaced by ETESTs®, performed as described at 2.4.1. In this case, the inoculum used was prepared, from the bacterial suspensions of each well of the 24-well plate.

In order to check for colony purity and discard possible contaminations during the assay, the content of each well was frequently plated in Columbia agar + 5% sheep blood plates, incubated at 37 ºC in 5% CO_2 for 24 h and analyzed using VITEK® MS system (bioMérieux).

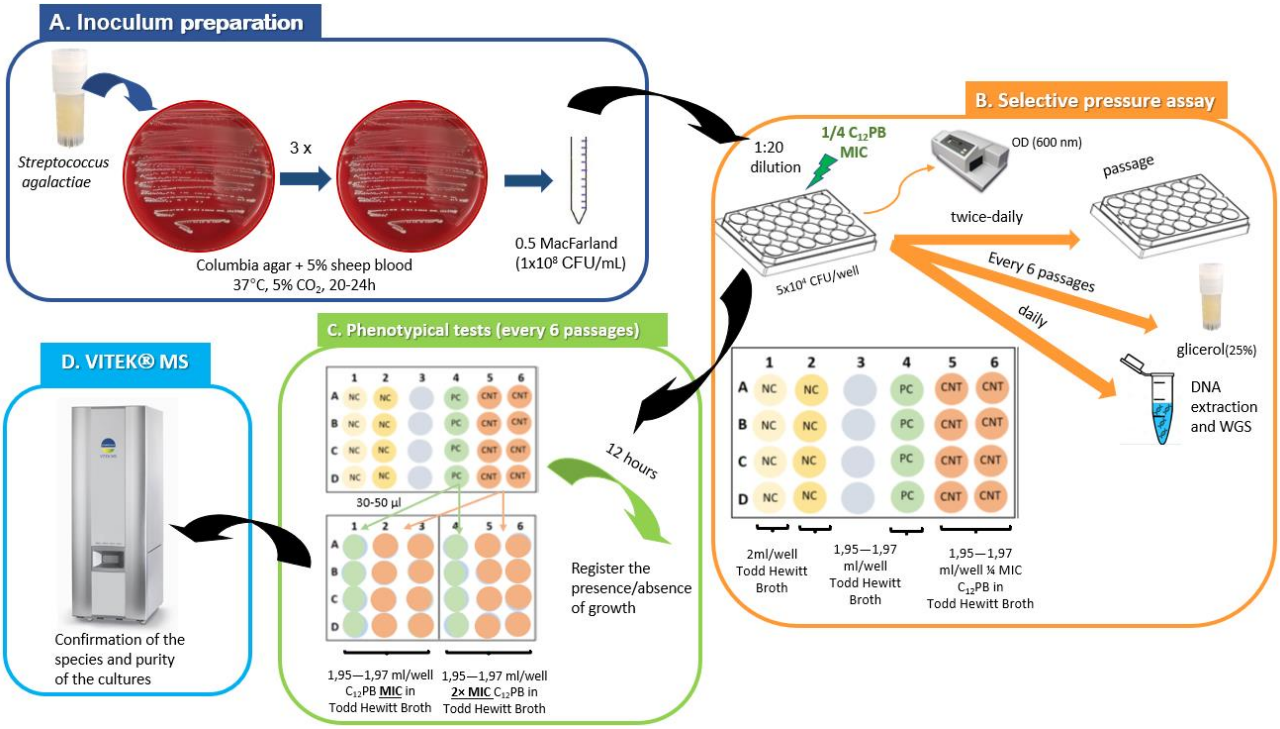

Figure 2.1. Flowchart displaying the experimental procedure of the selective pressure assay. The inoculum was prepared (**A**) to a final concentration of $5x10^5$ CFU/ml per well. **B-** 24-well plates were used to continuously propagate *S. agalactiae* populations under and without C_{12}PB/ERY pressure ($\frac{1}{4}$ MIC). NC- negative controls, PC- positive controls (without compound) and CNT- continuous pressure (with compound). Passages to a new plate were performed every 12 h and the content of each well was frequently collected in glycerol 25% and in 1.5 ml tubes for DNA extraction. **C-** methodology performed to evaluate the acquisition of resistance to the compound. To discard contaminations, the cultures were frequently analyzed on VITEK (**D**).

2.4.4 DNA isolation and WGS

S. agalactiae samples stored at ⁻ 20 ºC during the selective pressure assay (described in 2.4.3) were selected for DNA extraction. After thawing the samples, 400 µl of each were transferred to a new 1.5 ml tube and centrifugated at 14000 rpm for 10 min at 4°C. The pellet was resuspended in 200 µl of Tris-EDTA buffer containing 10 U mutanolysin (Sigma-Aldrich, St. Louis, USA) and 15 mg/ml lysozyme (Sigma-Aldrich, St. Louis, USA) and incubated for 2 h at 37 ºC, followed by digestion with proteinase K (Roche, Penzberg, Germany) for 1h30 at 56ºC. Genomic DNA was then extracted using the Isolate II Genomic DNA kit (Meridian Biosciences, Cincinnati, USA) according to the manufacturer's instructions. DNA concentration was assessed by fluorimetry using the Qubit™ dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, USA). DNA samples were then subjected to WGS using the Nextera XT Illumina library preparation protocol (Illumina, San Diego, USA), prior to paired-

end sequencing (2×250bp) on an Illumina MiSeq equipment (Illumina, San Diego,USA) available at the Portuguese National Institute of Health, according to the manufacturer's instructions.

2.4.5 Bioinformatic analysis

For the quality control and improvement of the reads, species confirmation (using the 8GB database available at https://ccb.jhu.edu/software/kraken/) and bacterial *de novo* assembly the INNUca v4.2.2 pipeline (https://github.com/B-UMMI/INNUca)[106] was used (fig 2.2). MLST prediction was determined using mlst v2.18.1 software (https://github.com/tseemann/mlst). Draft genome sequences were annotated using the RAST server v2.0 (http://rast.nmpdr.org/)[107] (fig 2.2).

Both for C_{12}PB and erythromycin, the identification of putative mutations and/or fluctuations in the accessory genome potentially responsible for AMR was made through comparative whole genome analysis of the clones propagated under C_{12}PB/ERY pressure *versus* the original ("ancestral") isogenic clone. The absence/presence of accessory genome was inspected through the alignment of *S. agalactiae* assemblies using the progressive algorithm of MAUVE v2.3.1 software (http://darlinglab.org/mauve/mauve.html)[108]. The presence of prophages was assessed with PHASTER web server (http://phaster.ca/)[109]. Additionally, the existence of putative plasmids was inspected using PlasmidFinder 2.1 (http://cge.cbs.dtu.dk/services/PlasmidFinder/) using default parameters (fig 2.2).

The identification of genomic markers associated with different levels of susceptibility to C_{12}PB/ERY as well as the intrinsic putative mechanisms of resistance was performed using Snippy v4.5.1 software (https://github.com/tseemann/snippy). Essentially, quality improved reads of the evolved populations under C_{12}PB or ERY pressure were individually mapped against the draft genome of the ancestral clone. Variants were called on sites that filled the following criteria: i) minimum mapping quality and minimum base quality of 20; ii) minimum number of reads covering the variant position ≥10; and iii) minimum proportion of reads differing from the reference of 90%. After discarding all variants developed from laboratory passaging, all SNPs and indels acquired under ERY and C_{12}PB pressure were carefully inspected and confirmed using IGV v2.11.0 (http://software.broadinstitute.org/software/igv/)[110].

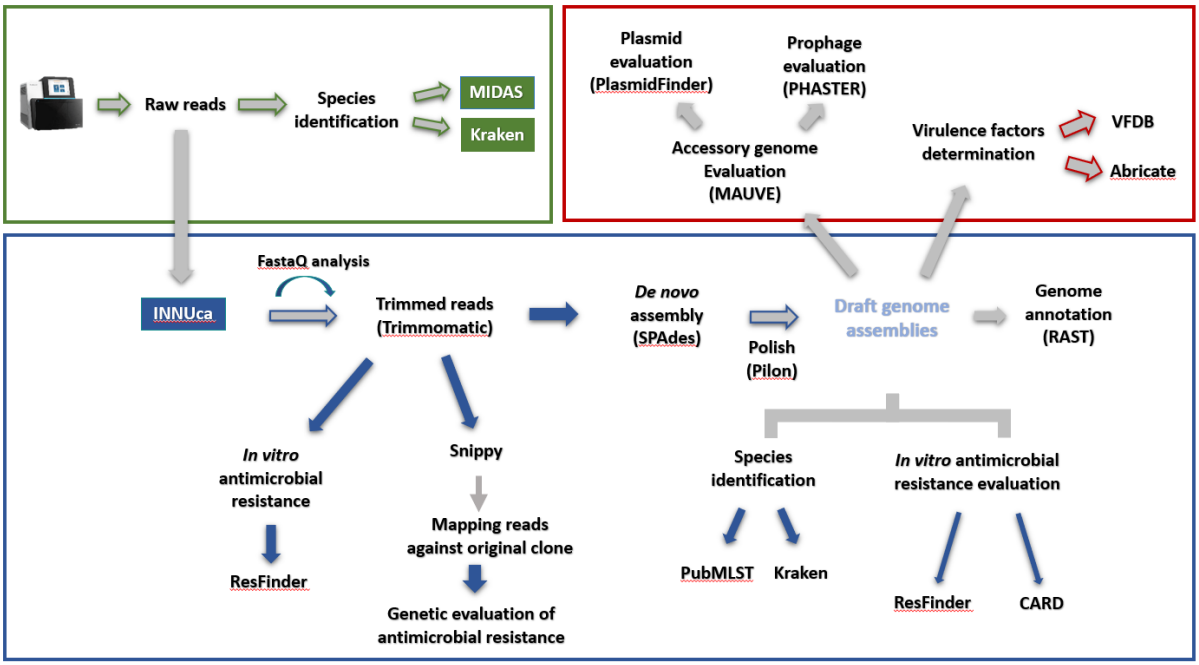

Figure 2.2. Flowchart displaying the steps of the bioinformatic analysis performed to identify the putative mechanisms of resistance on *S. agalactiae* populations exposed to C_{12}PB and ERY continuous pressure (sub-MIC conditions). The raw reads were originated from WGS of the bacterial populations exposed to these conditions.

2.4.6 Statistical analysis

Statistical analysis was carried out in Graphpad PRISM software, version 9.2.0. Statistical significance was assessed by two-way ANOVA test, with Tukey's post-hoc test. A P value <0.05 was considered statistically different: P< 0.05, *, significant; P< 0.01, **, very significant; P< 0.001, ***, P< 0.0001, ****, extremely significant.

3 RESULTS

3.1 Genomic characterization of the original clone (CL-1)

The clinical isolate (CL-1) used in the experiments (serotype V and ST19) was selected based on its clinical relevance: ST19 is one of the predominant colonizers in pregnant women and serotype V is one of the most prevalent serotypes in maternal colonizing strains in Europe[13] (particularly in Portugal)[13,31] and the most prevalent in Africa, where this problematic is more accentuated[24,27].

An analysis of the genomic markers present in CL-1 was performed in order to obtain a characterization of the initial clone as complete as possible. The presence of AMR genes was disclosed by querying the draft genome assembly against reference databases. The search revealed the presence of the *tetM* gene, which confers resistance to tetracycline, in accordance with the phenotypic resistance previously described (2.4). The presence of the *mprF* gene was also detected. This gene encodes for the Phosphatidylglycerol lysyltransferase and is known to be involved in *S. agalactiae* virulence and in resistance to cationic antimicrobial peptides (CAMP)[111]. Interestingly, the carefully analysis of the annotated genome revealed that the *tetM* gene was acquired through the acquisition of the transposon Tn916. The gain of resistance determinants to tetracycline through integrative and conjugative elements (ICEs), particularly Tn916, is a known mechanism in several bacteria including *S. agalactiae*[33,40].

3.2 Minimum Inhibitory Concentration

Firstly, the MICs for C_{12}PB and ERY were determined. The MIC of C_{12}PB was 12,5 µM. For ERY, two strategies were performed: i) broth microdilution and ii) Etest: using Columbia agar + 5% sheep blood (medium recommended by EUCAST[105]) and using Todd Hewitt Broth (medium used in all assays performed in this work). Only a slightly difference was observed between the two media: with Todd Hewitt broth the MIC was 0.125 µg/ml, both by Etest and microdilution while with the media recommended by EUCAST, the MIC was 0.19 µg/ml (fig 3.1). According to EUCAST breakpoints[112] (ERY Susceptibility - MIC $\leq$ 0.25 µg/ml), CL-1 was initially susceptible to ERY.

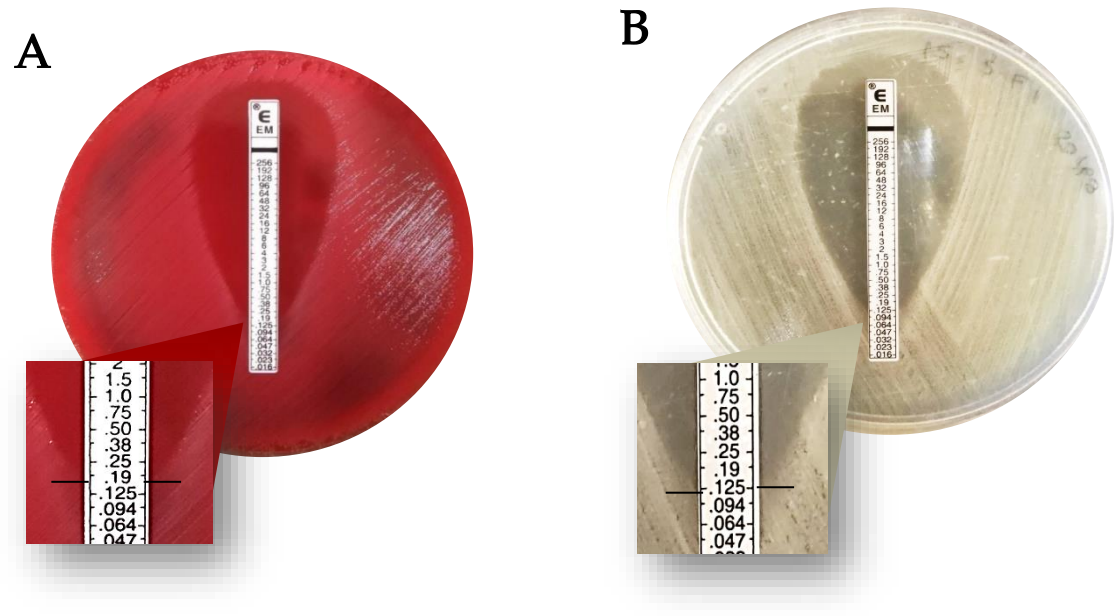

Figure 3.1. Determination of erythromycin MIC by Etest. A- Columbia agar + 5% sheep blood, **B-** Todd Hewitt Broth. The MIC value is where the ellipse edge of growth intersects the strip (indicated by the black lines).

3.3 Growth curves

Growth curves were performed in the presence of different concentrations of C_{12}PB and ERY (fig 3.2) to assess the following topics: i) the effect of C_{12}PB and ERY on the growth, in order to select a sub-mic that does not significantly compromise the strain's growth; ii) the duration of the exponential phase, in order to select an appropriate timepoint for the continuous passages; and iii) understand the growth behavior of the isolate, namely, the OD_{600nm} reached in each growth phase, in order to efficiently monitor the growth during the selective pressure assay.

The Lag phase occurred for 4 hours, followed by an exponential phase that was extended until about 10-12 hours from where stationary phase begins. Interestingly, C_{12}PB sub-MICs seems not to affect *S. agalactiae* growth since almost no differences were observed in the growth behavior between the C_{12}PB-exposure ($\frac{1}{2}$ MIC, $\frac{1}{4}$ MIC and $\frac{1}{8}$ MIC) and non-exposure (positive control) conditions (fig 3.2-A). In contrast, exposure to ERY sub-MICs affected the bacterial growth, which is particularly noticeable for ½ MIC, where the strain displayed an extended lag phase (between 0 and 10 h) and achieved lower cell densities (fig 3.2-B). As expected, for both compounds, no growth was observed at the MIC.

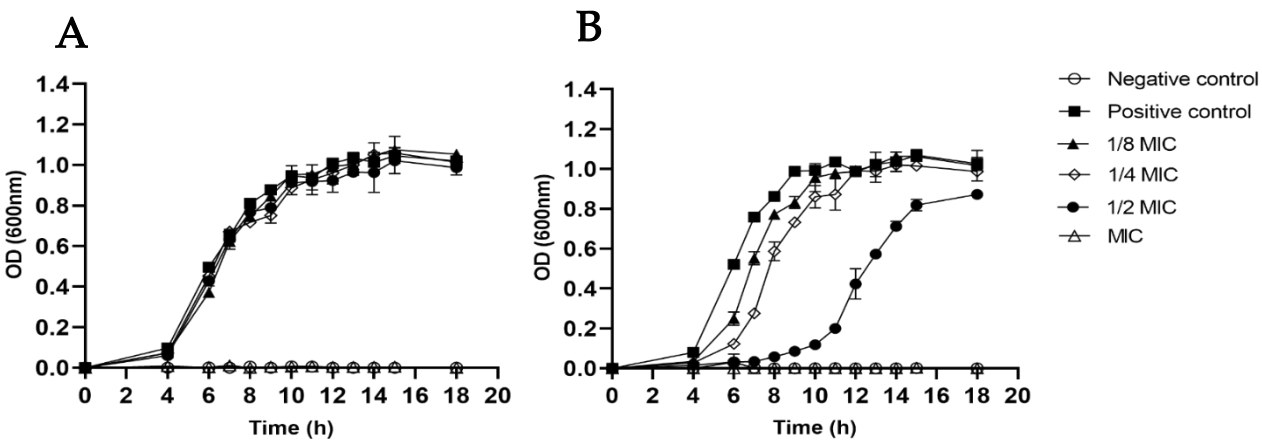

Figure 3.2. *S. agalactiae* growth curves in the presence of C_{12}PB (A) and erythromycin (B). Curves were determined without (positive control) and under different concentrations ($\frac{1}{8}$ MIC, $\frac{1}{4}$ MIC, $\frac{1}{2}$ MIC, and MIC) of each compound. Negative controls (broth solely) are also shown. Values plotted are expressed as mean ± SD.

3.4 Evaluation of antimicrobial resistance acquisition under selective pressure

Overall, both the *in vitro* selective pressure assays comprised a total of 56 passages. The evolution of the MIC during the assays is shown in figure 3.3. To identify the putative mechanism of resistance, or confirm at genomic level the absence of resistance, some of the replicates were selected for WGS, for both assays (fig 3.4). The selection was performed based on enough replicates to ensure a correct and representative analysis of the population genomic alterations during the selective pressure assays. For each replicate, only the passages where phenotypic alterations were observed (increase on the MIC) were sequenced, plus the initial clone and the endpoint.

No change in C_{12}PB susceptibility (MIC=12.5 µM) was observed during the whole assay (fig 3.3-A), which also confirmed at the genome level. Indeed, comparative genomic analyses between clones exposed to C_{12}PB *versus* the original isogenic clone revealed only two SNPs, namely c.1130A>T (p.D377V) and c.1220C>T (p.P407L), on the gene coding for guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase, or (p)ppGpp synthase II, each of these mutations appearing only in one replicate and passage.

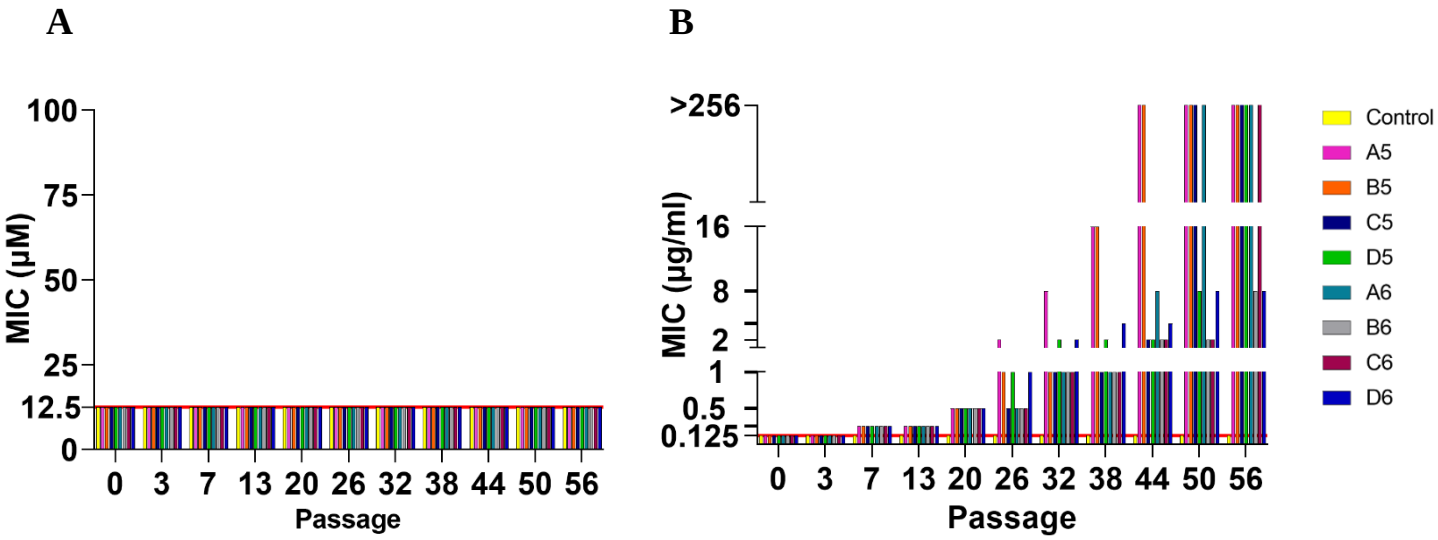

Figure 3.3. MIC evolution of *S. agalactiae* isogenic clones exposed to continuous C_{12}PB (A) and ERY (B) sub-MICs. MIC values in each passage are represented for the controls (propagated without compound) and for the eight replicates (A5-D5 and A6-D6) for each compound. The red line represents the baseline MIC.

In contrast, for ERY, the MIC doubled after only seven passages and continued to increase up to >256 µg/ml (>2000-fold the initial MIC) at the end of the assay (fig 3.3-B). Since there were eight replicates, the evolution of the MIC presented slightly differences between them (fig 3.3-B). For instance, A5 and B5 replicates reached MICs of more than 256 µg/ml at passage 44 whereas D6 replicate presented a slower MIC increase, achieving 8 µg/ml (corresponding to 64-fold the initial MIC) at passage 56. The differences between the replicates suggest the emergence of bacterial populations with different levels of susceptibility to erythromycin. Nevertheless, the rapid appearance of resistance was observed in all replicates as seen by the high-level resistance to ERY at the end of the assay

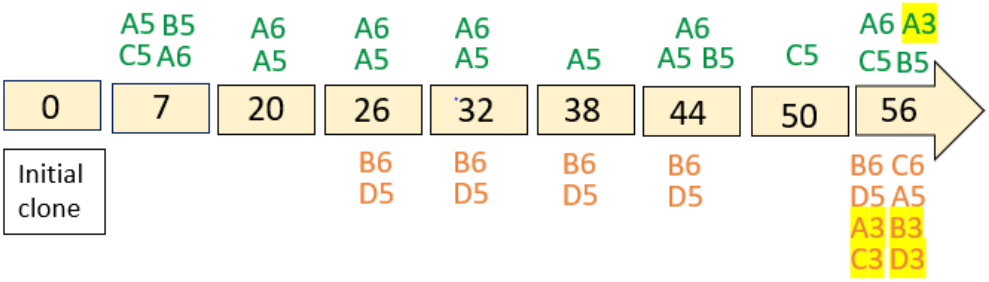

Figure 3.4. Representation of the replicates and passages subjected to WGS. The numbers represent the passage, replicates exposed to erythromycin and C_{12}PB are presented in green and orange, respectively. A3, B3, C3 and D3 (highlighted in yellow) are controls propagated without compound

Multiple mutations were identified on the clones propagated under erythromycin subinhibitory concentrations (table 2). Some of these mutations occurred in genes coding for proteins, or RNAs, known to be associated with erythromycin resistance acquisition, namely large subunit ribosomal protein L22, *23SrRNA* m^5U1939-methyltransferase and *23SrRNA*[56,57,113,114]. In addition, mutations on genes coding for proteins involved in regulatory and metabolic systems were also present[115,116] (table 2).

It is also interesting to refer that apart from the mutations described, a deeper genomic analysis of the populations exposed to C_{12}PB/ERY pressure, searching for resistance determinants and other features, did not revealed any addiction to the original clone. All populations preserve the *tetM* and *mprF* genes.

Of note, comparative genomic analysis of the clones propagated without surfactant or antibiotic versus the original clone revealed the following mutations: c.2188C>T (p.R730C) on a gene coding for a predicted glycogen debranching enzyme; c.454T>A (p.L152I) on the gene coding for the polysaccharide biosynthesis protein CpsM(V); and c.638C>A (p.A213E) on the gene coding for the lysyltransferase protein. These mutations were also present in some clones propagated under C_{12}PB/erythromycin pressure but were attributed to laboratory passaging and therefore discarded from the analysis.

Table 2. Mutations exhibited by resistant bacterial populations exposed to erythromycin sub-MIC.

Product	Alteration				Bacterial population (Passage)
	Type	DNA	Protein	Effect	
23S rRNA m^5U1939-methyltransferase	CDS	c.437_438insT	p.R147fs	Frameshift variant	A5 (P32, P44, P56)
23S rRNA	RNA	c.842T>C	-----	-----	A5 (P44), B5 (P56)
Large subunit ribosomal protein L22	CDS	c.274C>A	p.R92S	Missense variant	A6 (P20, P26)
	CDS	c.275G>A	p.R92H	Missense variant	A6 (P32, P44, P56)
	CDS	c.284G>A	p.G95D	Missense variant	A6 (P32, P44, P56), B5 (P44), C5 (P50, P56)
Transmembrane histidine kinase CsrS	CDS	c.1332T>A	p.D444E	Missense variant	A6 (P32, P44, P56)
Hypoxanthine-guanine phosphoribosyltransferase	CDS	c.401G>T	p.G134V	Missense variant	A6 (P32, P44, P56)
GMP synthase, ATP pyrophosphatase subunit	CDS	c.419G>A	p.S140N	Missense variant	B5 (P44), C5 (P56)

3.5 In vitro C_{12}PB toxicity and pro-inflammatory profile towards human epithelial cell lines

Antimicrobial compounds for topical applications must be able to prevent bacterial infections without causing significant damage towards host cells. Thus, C_{12}PB's toxicity and inflammatory potential towards *in vitro* models of the human vaginal mucosa were evaluated.

In vitro cytotoxicity was evaluated by assessing HeLa and Caco-2 cell viability after exposure to different concentrations of C_{12}PB for different timepoints. After exposure, the surfactant's capacity to induce inflammation in these cells was assessed by the quantification of a pool of inflammation biomarkers commonly used in vaginal microbicides candidates' studies[99-101].

Since C_{12}PB susceptibility varies among different isolates, and this fact will be considered to select the concentration of C_{12}PB present in the final product, it is important to evaluate the C_{12}PB toxicity in a range of concentrations bactericidal for different isolates. The interval between the dashed lines, in figure 3.5, indicates the concentrations (6.25 – 25 µM) corresponding to the MIC for different *S. agalactiae* isolates, previously calculated by Inácio *et al*.[98] .

For both cell lines, viability decreased with the increase of C_{12}PB concentration and exposure time. C_{12}PB was more toxic for Hela cells in comparison with Caco-2 cells (Fig 3.5). For Caco-2 cells, toxicity was only observed at concentrations near the CMC. At bactericidal concentrations to *S. agalactiae* (between dashed lines, fig 3.5-A), cell viability remained almost unaltered from the control (75.7 ± 2.9 – 96.3 ± 2.9 %) for all timepoints. The results were consistent with the absence of pro-inflammatory cytokines or chemokines verified (Appendix B, fig B.2).

For HeLa cells, a considerable reduction of cell viability due to C_{12}PB exposure was observed at concentrations much lower than the CMC, especially for the longest exposure times (180 and 360 min). Nevertheless, at bactericidal concentrations for *S. agalactiae* (between dashed lines, fig 3.5-B), no substantial loss in cell viability was observed for 20 and 60 min of incubation (cell viability varying between 81.4 ± 2.1 and 84.2 ± 2.6 %). For the longest exposure times, the decrease in HeLa cells viability was more accentuate, especially for 360 min at the highest C_{12}PB MIC (25 µM), where cell viability declines to ~ 47± 5 % of the control.

Regarding inflammation, for HeLa cells, only an increase of IL-6 and IL-8 was detected (fig 3.6). For both cytokines, inflammation was only observed 24 hours after C_{12}PB exposure as can be seen by the increase of IL-6 and IL-8 concentration in the supernatants collected 24 hours (fig 3.6-B and 3.6-D), but not immediately (fig 3.6-A and 3.6-C), after C_{12}PB exposure. Higher IL-6 concentrations are observed with the increase of C_{12}PB concentration and exposure time (fig 3.6-D). However, IL-6 levels were significantly increased ($P < 0.05$), compared with untreated cells, when the cells were exposed to all C_{12}PB concentrations for 360 minutes and to the two highest concentrations (CMC/500 and

CMC/154) for 180 minutes. Additionally, IL-8 was only increased at the highest concentration tested (CMC/154) for the longest incubation time (360 minutes). The quantification data of the remaining cytokines (IL1-β, IL-10, MCP-1, and MIP-1) released by HeLa and Caco-2 cells, as well IL-6 and IL-8 released by Caco-2 cells, is shown in appendix B (fig B.1 and fig B.2).

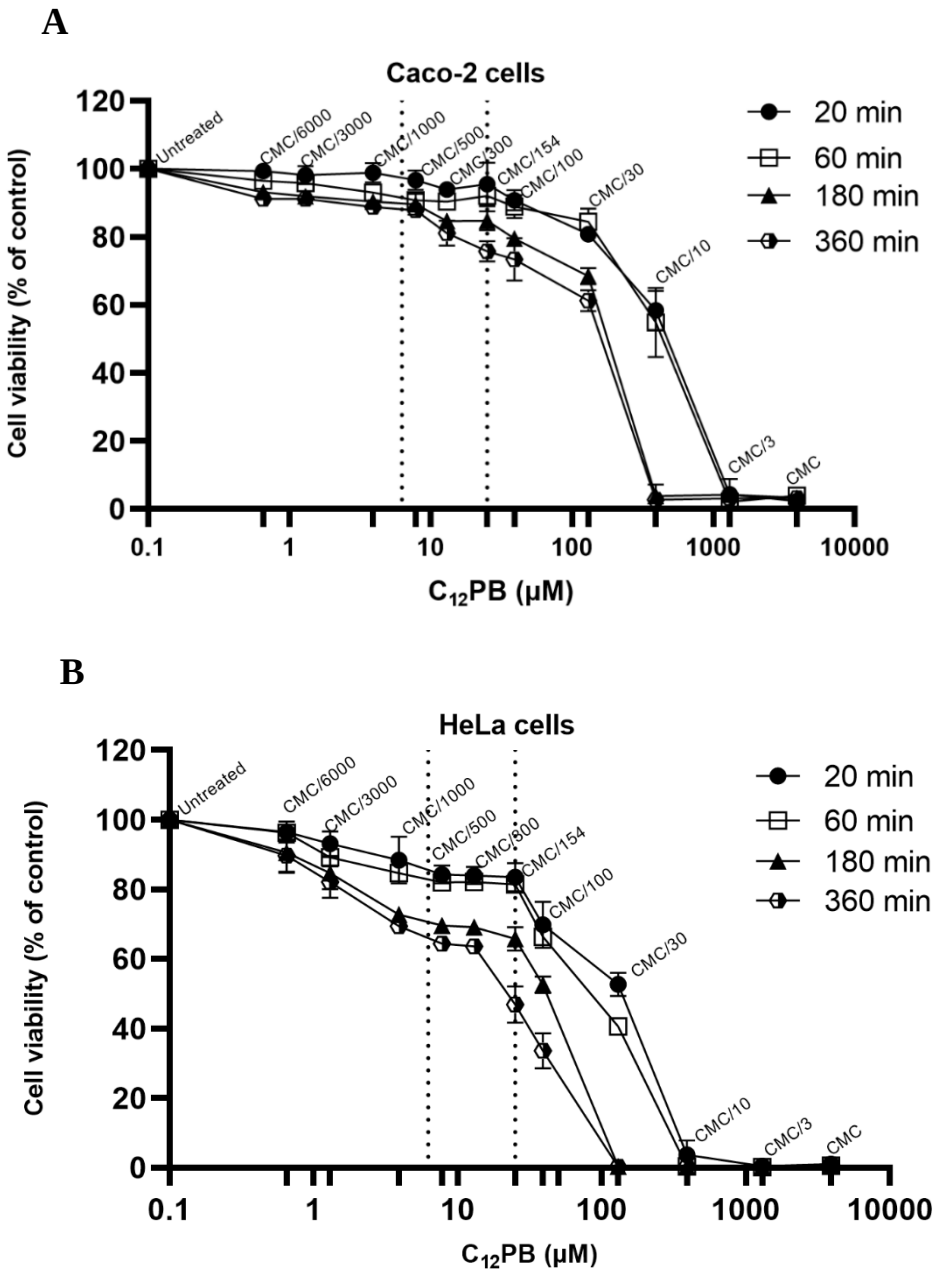

Figure 3.5. Concentration and exposure time dependence of the effects of C_{12}PB on HeLa (A) and Caco-2 (B) cell viability. Cells were exposed to concentrations of C_{12}PB corresponding to submultiples of the critical micellar concentration (CMC=3900 μM) during 20, 60, 180 and 360 minutes. Cell viability is expressed as % of control, which corresponds to untreated cells. Surfactant concentrations in the *x* axis are represented on a logarithmic scale. The interval between the dashed lines indicates the concentrations corresponding to the MIC for different *S. agalactiae* isolates, calculated by Inácio *et al*.[98]. Values plotted are expressed as mean ± SD of three independent experiments.

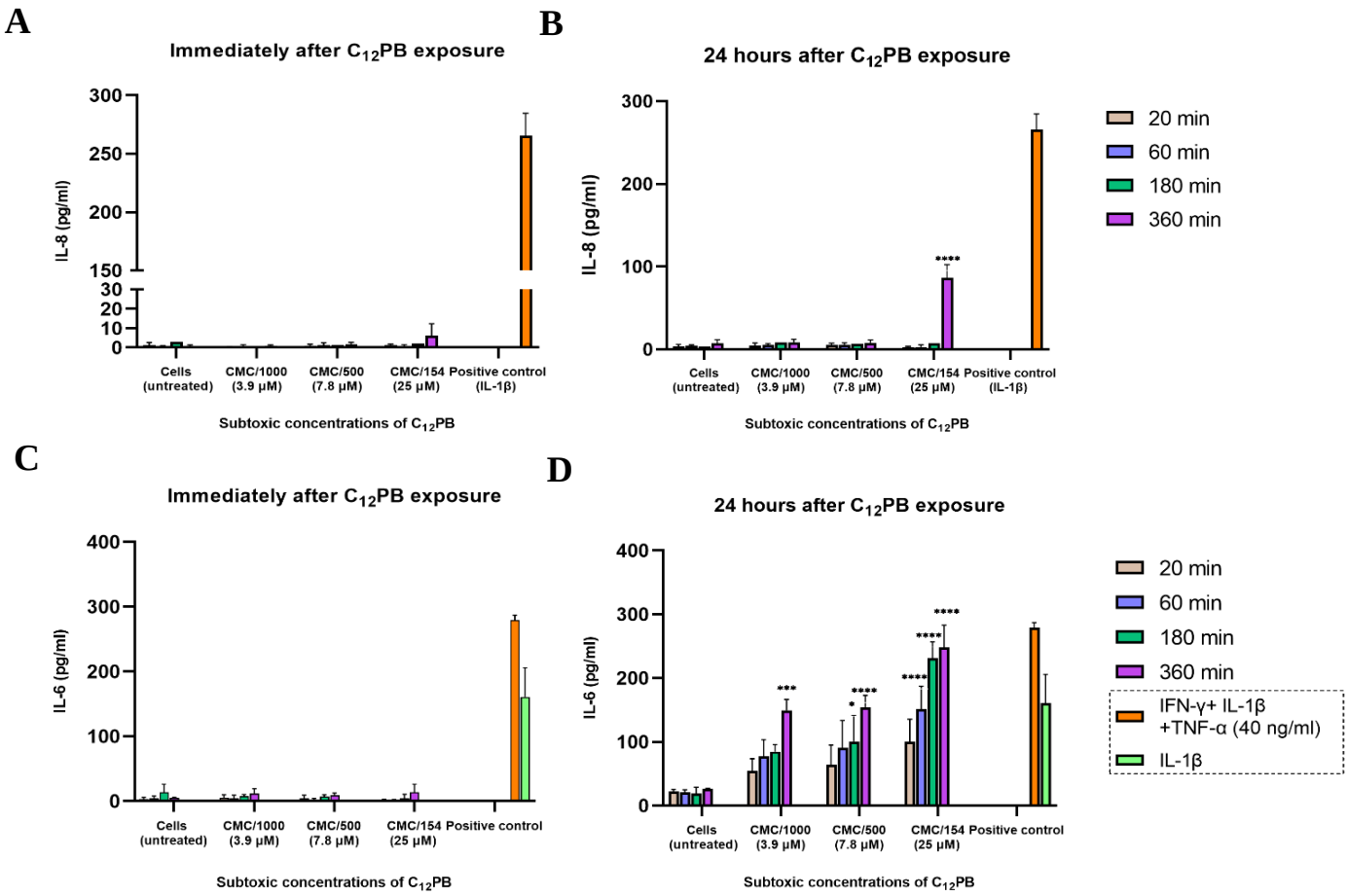

Figure 3.6. Effects of C_{12}PB on the secretion of inflammation biomarkers by HeLa cells. Quantification of IL-8 (A, B) and IL-6 (C, D) released by HeLa cells incubated with C_{12}PB for 20, 60, 180 and 360 minutes. For each cytokine, results are shown immediately after C_{12}PB incubation (A, C) and after 24 hours in surfactant free medium (B, D). Cell were incubated with three C_{12}PB concentrations, submultiples of the CMC (CMC/1000= 3.9, CMC/500= 7.8 and CMC/154= 25 µM), and one or two positive controls. Data is shown as mean +SD of three independent assays. Two-way ANOVA test (Tukey's post-hoc test): Asterisks (*, P< 0.05; ***, P< 0.001 and ****, P< 0.0001) signify differences from the respective control (untreated cells).

4
DISCUSSION

Although intrapartum antibiotic prophylaxis during labor and delivery has decreased the incidence of GBS early onset neonatal infections, this prevention approach still has many problems and is not well-established worldwide. Indeed, in 2020 *S. agalactiae* caused 91 000 newborn deaths and left 40 000 infants living with neurological impairment. Moreover, the WHO recently revealed that the global burden of *S. agalactiae* is higher than previously recognized[7,42].

In addition, the dramatic increase of AMR that has been observed in the last years is a major global health problem and the continuous use of antibiotics tends to aggravate this issue. The WHO recognized the development of a vaccine for *S. agalactiae* as a priority, but this task has been challenging and even though numerous vaccines candidates are being studied, some of them for years, there is no vaccine available[42]. Therefore, novel, and effective preventive and therapeutic measures are urgently needed, especially in under-developed countries where the largest burden of *S. agalactiae* is seen.

In the last years, QASs are re-appearing as very attractive microbicidal alternatives as primary chemical barriers to bacterial infections transmitted through skin or mucosal contact. Although antimicrobial resistance to QAS has already been described[94,95], no data seems to exist concerning C_{12}PB and GBS. The present study pretends to contribute to fill that gap, by evaluating C_{12}PB ability to induce AMR in a susceptible *S. agalactiae* clinical isolate, during an *in vitro* evolutionary window that mimics QAS prolonged use.

Results reported in this work showed that C_{12}PB has a very low potential of inducing antimicrobial resistance in GBS. In fact, C_{12}PB baseline MIC remained constant during the 56 passages of exposure to C_{12}PB ($\frac{1}{4}$ MIC) and no resistance-associated mutations were found at genomic level. It could be speculated that the appearance of resistance to C_{12}PB would be observed if the selective pressure assay was extended. However, the adapted "evolutionary in vitro selective pressure" approach showed to be effective to evaluate the acquisition of antimicrobial resistance in S. agalactiae. In fact, for erythromycin, the antibiotic used as control due to its use on IAP and to the known capacity for inducing antimicrobial resistance, a completely distinct scenario was observed. In a 56 passages window, the appearance of

phenotypic resistance to erythromycin was observed as early as the 7th passage and it was possible to follow the emergence of bacterial populations with different levels of susceptibility to erythromycin throughout the assay. In addition, the 56 passages window was enough to observe a dramatic increase on the MIC (from 0.125 to >256 µg/µl). Therefore, these results contribute to fill the gap on "evolutionary in vitro selective pressure" methodologies and data in *S. agalactiae.*

The acquisition of phenotypic resistance to erythromycin was corroborated at the genome level by the identification of resistance-associated mutations at ribosome level. Curiously, different mechanisms of erythromycin resistance were found among the replicates and, in some of them, more than one resistance-associated mutation was present. As an example, in clone A5 resistance seemed to be the resultant from the mutation on the gene coding for 23S rRNA m^5U1939-methyltransferase, while in clone B5 seems to be due to the mutation on the gene coding for large subunit ribosomal protein L22. Yet, both clones exhibited the same mutation on the 23S rRNA, known as a target for mutations causing resistance to macrolides[114]. Nevertheless, these dissimilarities may be merely due to a bottleneck phenomenon during passages of each population.

Of note, it was also interesting to note that the some of the replicates exposed to erythromycin (namely the ones with mutation on ribosomal protein L22) exhibited some mutations that, according with literature, were not associated with resistance. These mutations occurred in proteins involved in purine metabolism or in regulation of gene expression. These are important systems and pathways involved in bacterial survival, virulence, and pathogenesis[115-117]. Therefore, the appearance of these mutations alongside with the resistance-associated mutations could possibly be a compensation for the loss of fitness commonly verified in erythromycin resistant mutants, with resistance conferred by mutations affecting the ribosome[56].

In addition, the only mutations identified on the clones exposed to C_{12}PB, although they were not shared among clones nor maintained along the passages, were on the gene coding for (p)ppGpp synthetase II. This protein is involved in bacterial stringent response, a ubiquitous stress signaling pathway activated in nutrient starvation and other stress conditions[118]. Although C_{12}PB sub-MICs seem not to significantly affect bacterial growth, the presence of this compound in the propagation media during a prolonged period could possibly induce some stress on the bacteria.

Besides being effective against the target microbes, a microbicide must be safe for the target tissue. Importantly, this was exactly the reason of the failure of the attempts to validate surfactants as prophylactics for STIs. Studies have discussed that these attempts may have failed to consider the effect of the surfactant concentration and thus, the disappointing results were due to the use of an excessive concentration of surfactant. For instance, in the study of Nonoxynol-9 (N-9)[87,88,91], a nonionic surfactant contraceptive, N-9 was used in the phase III of clinical trials at concentrations ranging from 5 to 100 mg/ml which is much above its CMC (0.05 mg/ml)[91,93]. At concentrations near or above the CMC,

surfactants are known to act indiscriminately, disrupting both microbial and mammalian cells membranes. In this work, this concentration/toxicity dependence was considered.

Concerning the use of surfactants for topical application, epithelial cells are the first to encounter the compound. Vaginal and rectal epithelial cells are known to be affected by surfactants, especially vaginal columnar epithelium. Hence, it is extremely important to assess the toxicity and pro-inflammatory potential of different surfactant's concentrations towards those cells, to assure its safety as a prophylactic microbicide candidate.

In this work, *in vitro* susceptibility and pro-inflammatory effects of C_{12}PB were screened in two types of cells, resembling the types of cells existing on the target tissue. Caco-2 cell line, a laboratory model of mammalian columnar epithelium that is fully polarized and grown to confluence was used. In addition, HeLa cells were used as a model for non-polarized but fully confluent vaginal cells. It was showed that at concentrations bactericidal for *S. agalactiae*, C_{12}PB is not toxic nor induces inflammation towards Caco-2 cells. Differently, some degrees of toxicity and inflammation were registered for HeLa cells, particularly for the highest concentrations and exposure times. These variations are probably due to the intrinsic differences between these two types of cells. Confluent fully polarized Caco-2 cells, exhibit a highly ordered apical membrane with well-formed tight-junctions, making these cells substantially more resistant compared to non-polarized HeLa cells[96,119].

Regarding C_{12}PB pro-inflammatory profile, only IL-6 and IL-8 were increased, which is in accordance with the fact that these cytokines are commonly increased in cervicovaginal lavage and swab samples of microbicides candidates[99-101]. Interestingly, the findings regarding inflammation (detected only after 24h of C_{12}PB exposure) were in accordance with previous studies showing that cationic surfactants toxic effects, when used at concentrations below the CMC, are more subtle and act at the intracellular level compared to the rapid damage, with membrane destruction, observed at higher concentrations[119]. This mode of toxicity could possibly explain the late inflammation observed, only after the toxic effects had time to occur and induce the release of pro-inflammatory cytokines.

5

CONCLUSIONS AND PROSPECTS

In conclusion, results obtained in this work showed that together with its antimicrobial activity, C_{12}PB has overall low cytotoxic and pro-inflammatory potential and present a very low potential of inducing antimicrobial resistance in *S. agalactiae*.

It is important to note that the toxicity and inflammation results presented here were obtained *in vitro*. It is likely that in the complexity of the human vagina environment, with an acidic pH, biological fluids, vaginal microbiota and the cervicovaginal mucus barrier, the toxicity and inflammation observed in HeLa cells will be different, probably more subtle, or even absent. Thus, future *in vivo* studies will be crucial to assess C_{12}PB safety.

The next step will be the incorporation of C_{12}PB in a *in situ* delivery system. Polymeric hydrogels seem to be a promising option for a sustained release of the compound, allowing a significant decrease of the maximum C_{12}PB concentration in contact with the vaginal cells and thus adverting the potential toxic effects[53,54]. The data obtained in this study, along with future studies on the delivery vehicle properties, will be fundamental to select the appropriate C_{12}PB concentration to use, since it must satisfy at least two conditions: i) to be bactericide against S. agalactiae and ii) to be non-toxic or sub-toxic towards the vaginal epithelial mucosa.

The results described here, together with the previous findings highlight a C_{12}PB as promising candidate for the prevention of vertically transmitted *S. agalactiae* diseases. If the next tasks present positive results, the C_{12}PB-containing hydrogel could possibly be studied in clinical trials and hopefully become an available option. This prospect would be a great option especially in under-developed countries and would contribute to minimize not only the global burden of *S. agalactiae* but also the problematic of antimicrobial resistance.

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A

APPENDIX- OPTIMIZATION ASSAYS

A.1 Growth Conditions optimizations

To select the appropriate growth conditions for the selective pressure assay, growth curves were performed as described at 2.4.2, in parallel with Todd Hewitt broth and Cation-Adjusted Muller-Hinton broth + 5% defibrillated sheep blood, using two initial inoculum dilutions, 1:20 and 1:50. MICs for C_{12}PB were determined, as described in 2.4.1, in parallel with Todd Hewitt broth, Muller-Hinton Broth and Cation-Adjusted Muller-Hinton broth + 5% defibrillated sheep blood. After selecting Todd Hewitt broth as the appropriate media to use MICs were determined, for erythromycin and C_{12}PB, in parallel using different incubation conditions: 35 ºC ambient air and 37 ºC in 5% Co_2, in three independent assays.

A.2 Preliminary selective pressure assays

Multiple preliminary selective pressure assays were performed in order to optimize the volume of inoculum transferred during the passage and incubations conditions. 24-well plates were prepared as described at 2.4.3 and incubated at 35 ºC ambient air and at 37 ºC in 5% Co_2. After 12 hours, ODs at 600 nm were measured and registered and volumes between 30-50 µl were passed from each well to a new plate. This procedure was repeated for a minimum of three days up to two weeks for the different preliminary assays. The ODs values resulting from the different condition tested were compared and analyzed.

A.3 Phenotypical test optimizations

During the preliminary assays, different strategies were tested to select the most appropriate and effective to evaluate the acquisition of antimicrobial resistance through to selective pressure assay.

A.3.1 Agar dilution

For the C_{12}PB selective pressure assay, spots of 10 µl from each well on the 24-well plate were inoculated into Todd agar plates incorporated with different surfactant concentrations [¼ MIC (1.5625 µM), ½ MIC (3.125 µM), MIC (6.25 µM) and 2×MIC (12.5 µM)] and into todd agar plates without C_{12}PB as a positive control. The plates were incubated at 35 ºC, after 24 h the presence or absence of growth was registered.

A.3.2 Disk diffusion

For the erythromycin selective pressure assay, disk diffusion assay was tested for different incubation conditions. Bacterial suspensions from each well were adjusted to 0.5 McFarland and used to inoculate todd agar plates using a cotton swab. Antimicrobial susceptibility disks were prepared with different erythromycin concentrations [¼ MIC (0.00390625 µg/ml without CO_2; 0.03125 with 5% CO_2), ½ MIC (0.0078125 µg/ml without CO_2; 0.0625 µg/ml with 5% CO_2), MIC (0.015625 µg/ml without CO_2; 0.125 µg/ml with 5% CO_2) and 2×MIC (0.03125 µg/ml without CO_2; 0.25 µg/ml with 5% CO_2)] by impregnating the disks with 10 µl from each erythromycin solutions. The 4 disks were placed onto each plate and the plates were incubated at 35 ºC ambient air and 37 ºC in 5% CO_2. After 24 hours the inhibition or presence of growth for each disk and plate was observed and registered.

A.3.3 Broth microdilution

The broth microdilution method was tested for both C12PB and erythromycin. 96-well plates were prepared with concentrations corresponding to ¼ MIC, ½ MIC, MIC and 2×MIC (listed in A.3.2, without CO_2). Bacterial suspensions from each well were adjusted to 0.5 McFarland, diluted 1:20 in Todd Hewitt Broth and added to the respective wells, with the four different concentrations, from the 96-well plate (final concentration of 5×10^5 CFU/ml). Broth was used as negative control and broth + bacteria as a positive control. The plates were incubated at 35 ºC for 20-22 h.

A.3.4 Broth macrodilution

The broth macrodilution method was performed for C_{12}PB and erythromycin, two incubation conditions were tested for both: 35 ºC ambient air and 37 ºC in 5% Co2. Surfactant/erythromycin solutions (concentrations listed in A.3.2) were prepared in Todd Hewitt broth with a final volume of 20 ml, in 50 ml tubes. For each well from the 24-well plates, 10 µl were used to inoculate each one of the 8 tubes (¼MIC, ½ MIC, MIC, and 2×MIC, with and without CO_2). The tubes were incubated at 35 ºC ambient air and 37 ºC in 5% CO_2. After 12 h the tubes were observed, ODs at 600 nm measured, the absence of presence of growth was registered and the tubes were incubated again for more 12 hours, when the final observations and registrations were made.

A.3.5 24-well plates

The method described at 2.4.3.1, was tested with two different incubation conditions, and MIC and 2×MIC solutions were prepared accordingly (concentrations listed A.3.2). The plates were incubated 35 ºC ambient air and 37ºC in 5% Co2 and the presence or absence or growth was evaluated by measuring the OD at 600 nm after 12 and 24 h.

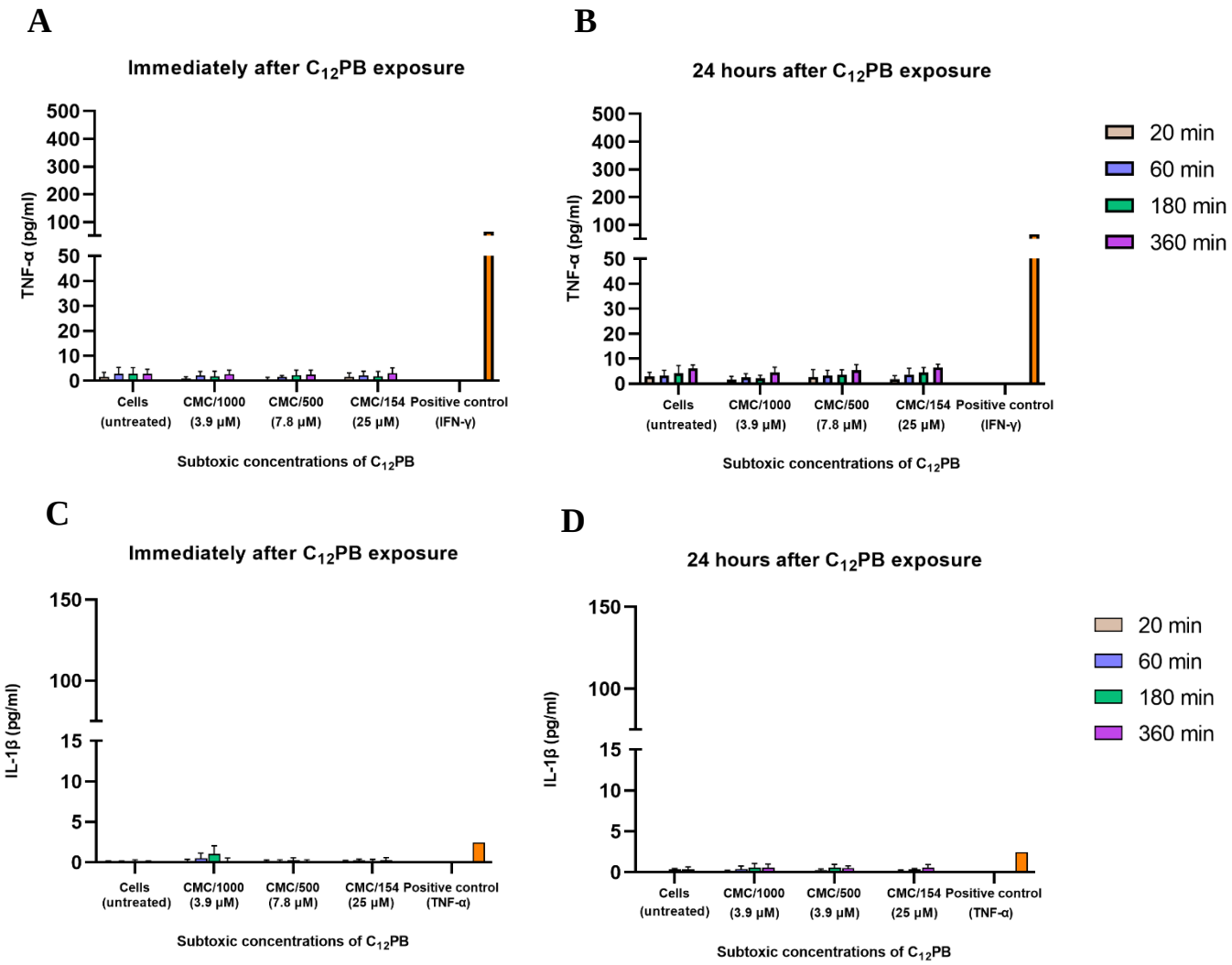
A.4 Erythromycin stability assessment

The stability of erythromycin ¼ 1/4 MIC solution (100× concentrated) was unknow and was therefore assessed, to make sure of always use an effective solution throughout the experimental assays. Briefly, erythromycin 3.125 µg/ml solutions were prepared and kept at 4 ºC and at ⁻ 20 ºC. After one week, the MIC for isolate CL-1 was determined (as described in 2.4.1) in parallel using the 2 solutions kept for one week, and a freshly prepared solution. The results for the 3 conditions were compared and analyzed.

B

Appendix- Cytokines and chemokines quantification results

B.1 Cytokines and chemokines released by HeLa cells



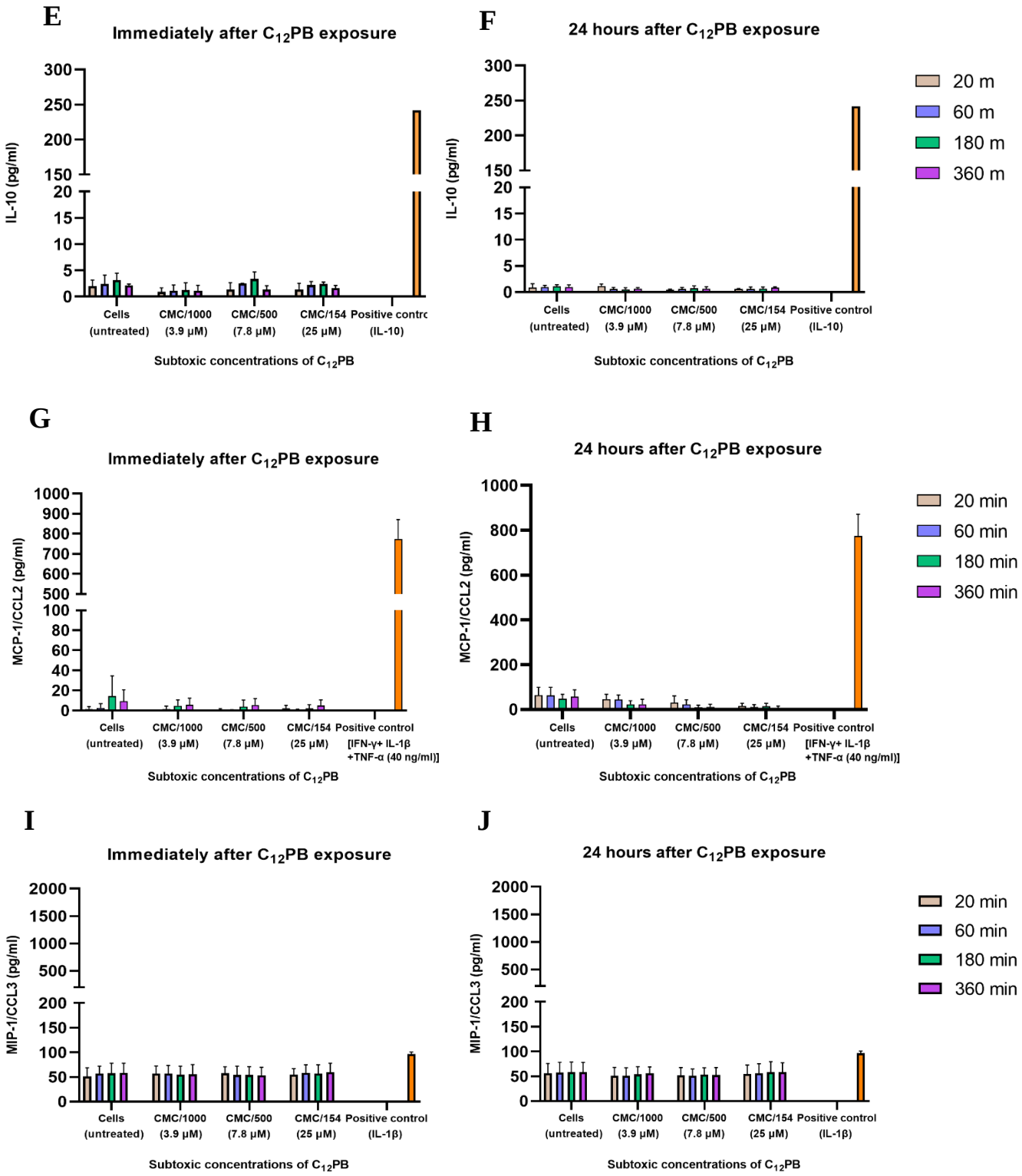
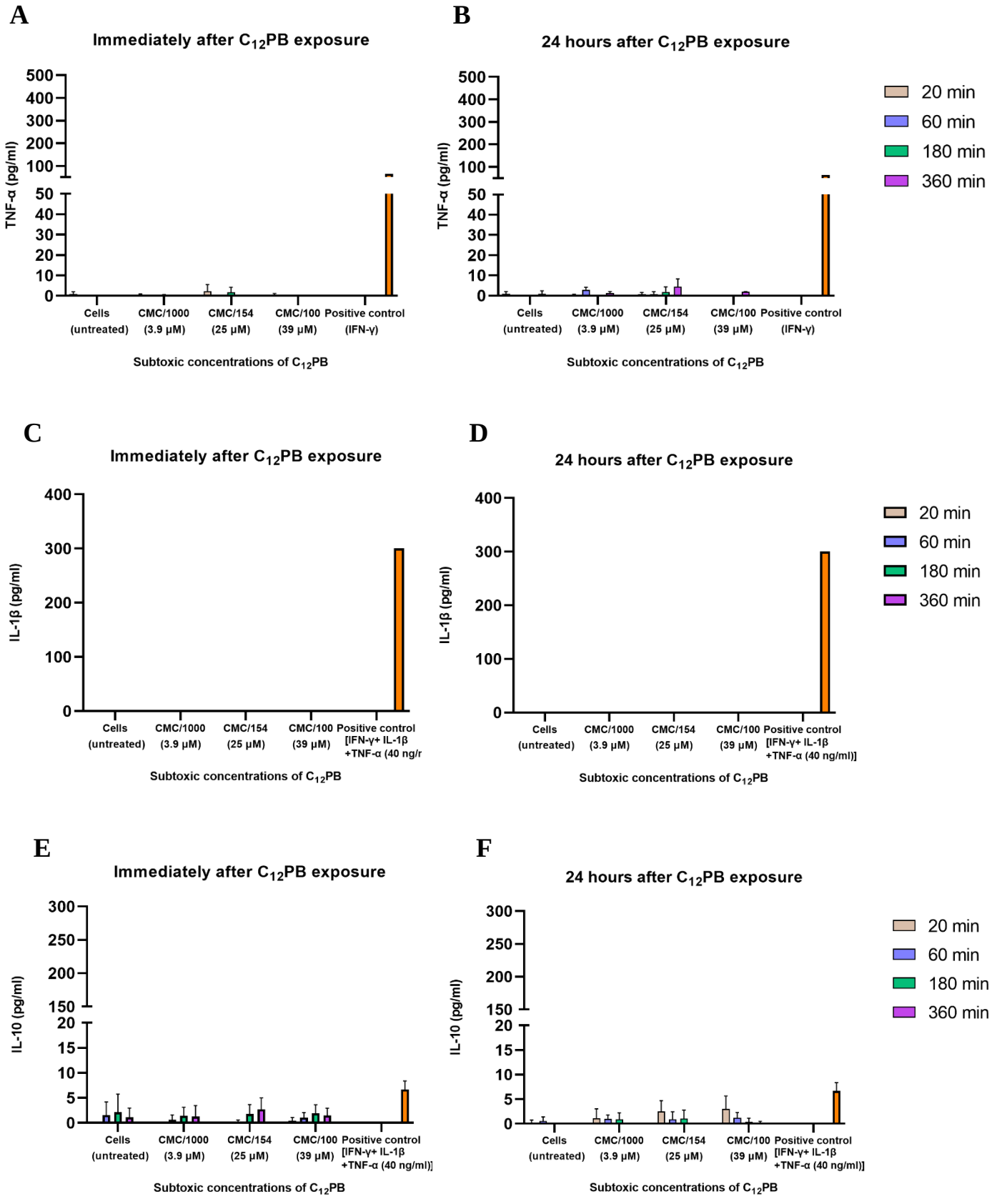
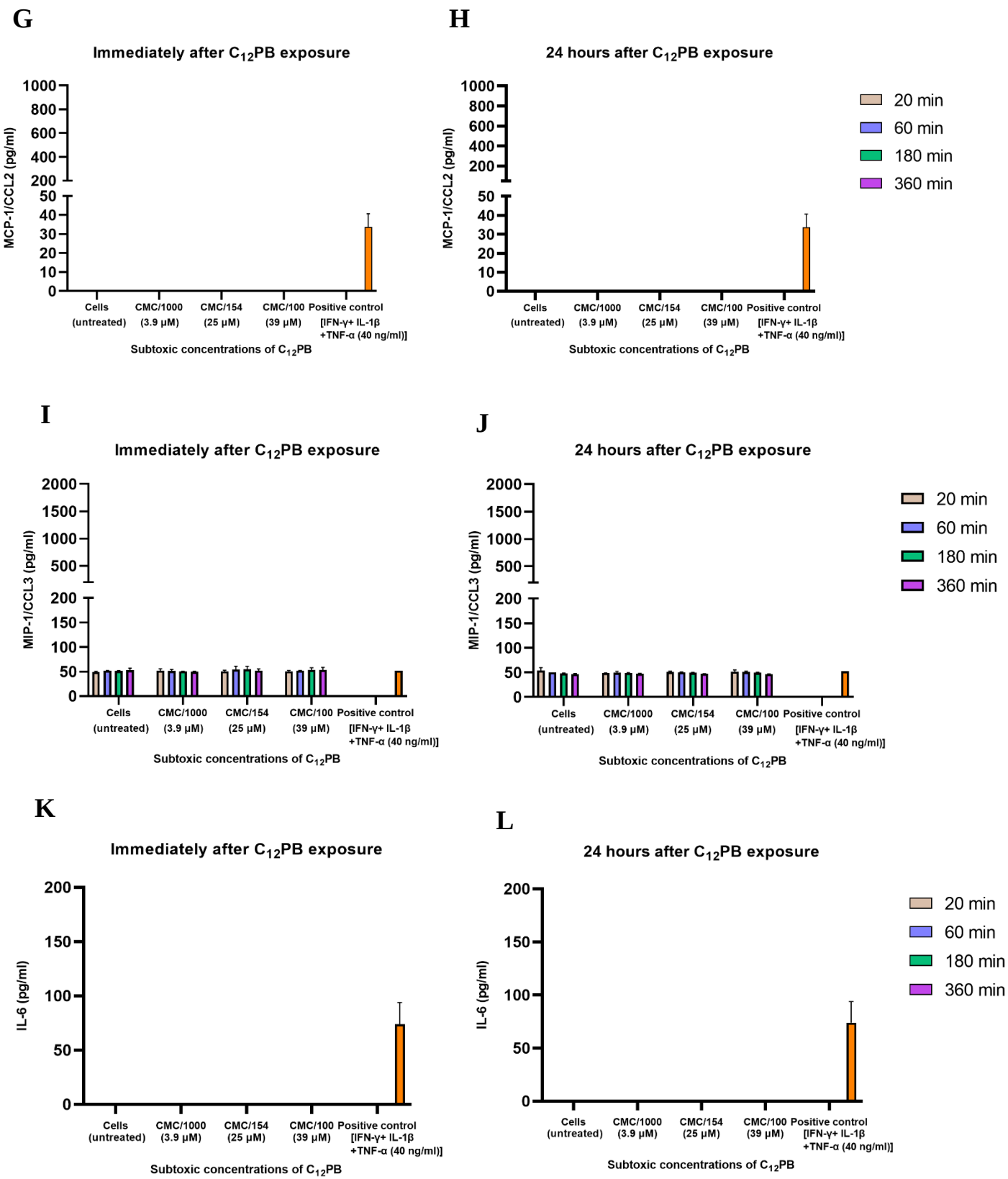


Figure B.1. Quantification of TNF-α (A, B), IL1-β (C, D), IL-10 (E, F), MCP-1 (G, H) and MIP-1 (I, J) released by HeLa cells incubated with C_{12}PB for 20, 60, 180 and 360 minutes. For each cytokine and chemokine, results are shown immediately after C_{12}PB incubation and after 24 hours in surfactant' free medium. Cells were incubated with three C_{12}PB concentrations (3.9, 7.8 and 25 µM) and a positive control.

B.2 Cytokines and chemokines released by HeLa cells




G
Immediately after C_{12}PB exposure
MCP-1/CCL2 (pg/ml)
1000
800
600
400
200
50
40
30
20
10
0
Cells (untreated)
CMC/1000 (3.9 µM)
CMC/154 (25 µM)
CMC/100 (39 µM)
Positive control [IFN-γ+ IL-1β +TNF-α (40 ng/ml)]
Subtoxic concentrations of C_{12}PB
H
24 hours after C_{12}PB exposure
20 min
60 min
180 min
360 min
I
Immediately after C_{12}PB exposure
MIP-1/CCL3 (pg/ml)
2000
1500
1000
500
200
150
100
50
0
J
24 hours after C_{12}PB exposure
K
Immediately after C_{12}PB exposure
IL-6 (pg/ml)
200
150
100
50
0
L
24 hours after C_{12}PB exposure

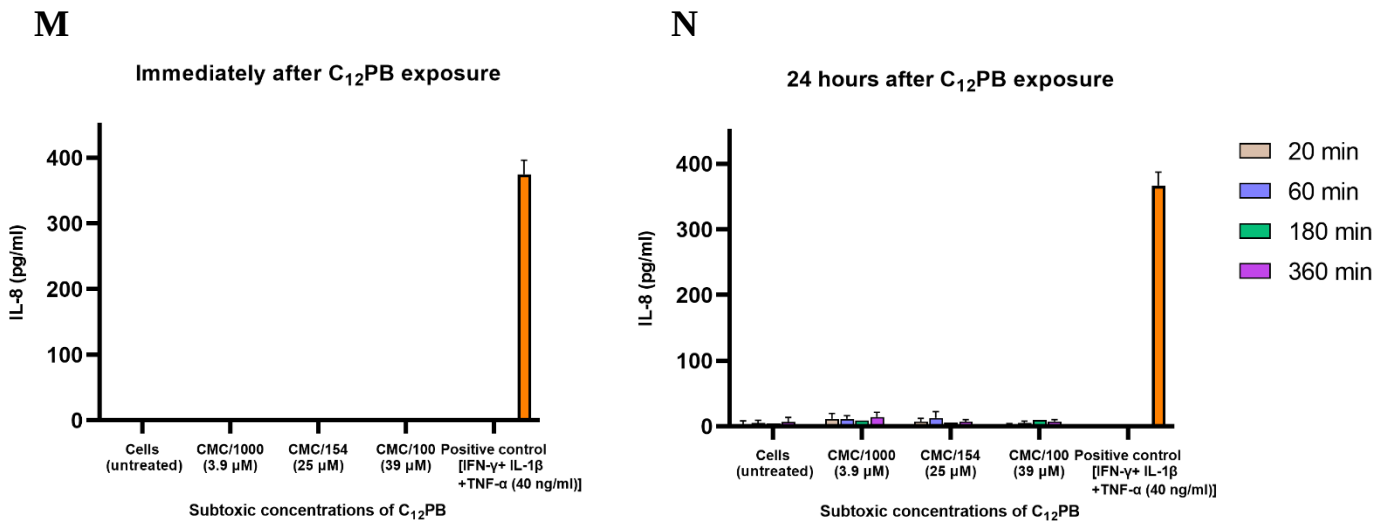


Figure B.2- Quantification of TNF-α (A, B), IL1-β (C, D), IL-10 (E, F), MCP-1 (G, H), MIP-1, (I, J) IL-6 (K, L) and IL-8 (M, N) released by Caco-2 cells incubated with C_{12}PB for 20, 60, 180 and 360 minutes. For each cytokine and chemokine, results are shown immediately after C_{12}PB incubation and after 24 hours in surfactant' free medium. Cell were incubated with three C_{12}PB concentrations (3.9, 25 and 39 µM) and a positive control.