TÉCNICO LISBOA

Influence of the Cell Membrane on Bacterial Persistence

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RESUMO

Este trabalho teve como objectivo estudar o aparecimento de células bacterianas persistentes em populações expostas a antibióticos e o efeito dos antibióticos na composição de ácidos gordos (FA) e potencial zeta (ZP) dessas células. Foram estudadas duas bactérias Gram-postitivas: *Staphylococcus aureus* e *Rhodococcus erythropolis*. A tolerância das células persistentes a vancomicina e teicoplanina, que inibem a biosíntese do peptidoglicano da parede celular, foi monitorizada pela contagem de unidades formadoras de colónias. Para isso, bactérias recolhidas na fase exponencial de crescimento foram expostas a elevadas concentrações de antibióticos: 50 e 100 $\mu g.mL^{-1}$. Cinéticas de morte bifásica, típicas do enriquecimento em células persistentes, foram observadas em ambas as espécies e condições testadas. Foram ainda obtidos padrões distintos por FTIR de células normais e persistentes. A dinâmica populacional das células persistentes sobreviventes após remoção do antibiótico, analisada por espectrofotometria a 600 nm, revelou repopulação da cultura, confirmando células persistentes como variantes fenotípicas reversíveis das células normais. A análise dos ésteres metilados de FA mostrou que ambas as bactérias respondem através do aumento do número de FA saturados e da diminuição significativa do número de FA ramificados. No caso do ZP, os valores diminuíram com o tempo de exposição e concentração dos antibióticos. Estes resultados sugerem claramente que as bactérias ultrapassam a acção dos antibióticos através da reorganização da membrana celular, de modo a reduzir a sua permeabilidade. Adicionalmente, ensaios de actividade enzimática e de consumo de oxigénio em células persistentes sugerem que as células estão activas em vez de 'adormecidas', refutando hipóteses anteriores.

Palavras-chave: *Staphylococcus*, *Rhodococcus*, vancomicina, teicoplanina, FAMEs, persistência

ABSTRACT

This work aimed at studying the appearance of persister bacterial cells in antibiotic exposed populations and the effect of antibiotics on the fatty acid (FA) composition and membrane zeta potential (ZP) of those cells. Two Gram-positive bacteria were studied: *Staphylococcus aureus* and *Rhodococcus erythropolis*. The appearance of persister cells tolerant to vancomycin and teicoplanin, which act by inhibiting cell wall peptidoglycan biosynthesis, was assessed by colony-forming unit counts. For that, bacteria in exponential growth phase were challenged with high antibiotic concentrations: 50 and 100 $\mu g.mL^{-1}$. Biphasic killing kinetics, typical for persister cell enrichment, were observed in both species and for the two tested antibiotics/concentrations. Furthermore, distinct Fourier transform infrared (FTIR) patterns were obtained for regular and persister cells. Population dynamics analysis of the survivor persisters upon antibiotic removal, monitored spectrophotometrically at 600 nm, revealed culture repopulation, confirming persister cells as reversible phenotypic variants of regular cells. Analysis of fatty acid methyl esters (FAMEs) showed that both studied bacteria respond to antibiotics by increasing the percentage of saturated FA while reducing significantly that of branched FA. Besides, the cells decreased the ZP with both time of exposure and antibiotic concentration. Together, these results strongly suggest that bacteria overcome antibiotic action by reorganizing the cell membrane, in order to reduce its permeability to the stress agent. Additionally, enzymatic activities and oxygen consumption rates of persister cells further suggest that they are active rather than 'dormant', refuting previous hypotheses.

Keywords: *Staphylococcus*, *Rhodococcus*, vancomycin, teicoplanin, FAMEs, persistence

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LIST OF ABBREVIATIONS

ca.	*circa* or approximately
CF	Cystic fibrosis
CLSI	Clinical and Laboratory Standards Institute
COPD	Chronic obstructive pulmonary disease
DAP	Diaminopimelic acid
e.g.	*exempli gratia* or for example
EPM	Exopolymeric matrix
EPS	Extracellular polymeric substances
FA	Fatty acid
FACS	Fluorescence activated cell sorting
FAMEs	Fatty acid methyl esters
FTIR	Fourier transform infrared
GFP	Green fluorescence protein
GC	Gas chromatography
hip	High-level persistence
i.e.	*id est* or in other words
LB	Luria-Bertani broth
MHB	Mueller Hinton broth
MIC	Minimal Inhibitory Concentration
NIH	*National Institutes of Health*
PAH	Polycyclic aromatic hydrocarbon
PCD	Programmed cell death
PDMS	Polydimethylsiloxane
PUFA	Polyunsaturated fatty acid
rpm	Rotations per minute
TA	Toxin–antitoxin
t_d	Duplication time
Tn	Transposon
TSA	Tryptic soy agar
TSB	Tryptic soy broth
WHO	*World Health Organization*
ZP	Zeta Potential

1. INTRODUCTION

The problem, and therefore the associated studies, of acute infections caused by pathogenic bacteria are not recent. Instead, this class of infections has been intensively studied for more than 100 years (Bjarnsholt, 2013). In recent years, with the advances obtained in biochemical and medical/pharmaceutical studies, these infections have been fought and effectively prevented by the development of modern vaccines, antibiotics and infection control measures. Until the last few years, most research into bacterial pathogenesis focused on acute infections, but these studies have been complemented by a new category of chronic infections caused by bacteria growing in slime-enclosed aggregates known as biofilms (Costerton *et al.*, 1999). Particular attention has been giving to a fraction of cells, called persister cells, which manage to remain alive even when the large, highly susceptible majority of biofilm-contained cells and/or planktonically grown cells are killed (Lewis, 2001). Although reduced, the persister fraction is suggested to be responsible for the recalcitrance of chronic infections which lead to recurrent therapy failures.

Staphylococcus aureus is a Gram-positive bacterium that has been widely implicated in many chronic infections, including those in cystic fibrosis patients and related to non-healing wounds of diabetes, chronic lung infections (*e.g.*, chronic obstructive pulmonary disease (COPD)) (Brusselle, 2011) and device/implant-related infections (Costerton *et al.*, 1999; Grant and Hung, 2013; Singh *et al.*, 2009). Giving their associated recalcitrance such infectious diseases are often untreatable (Lewis, 2010). On the other hand, the study of *Rhodococcus erythropolis* has proved of extreme importance due to its applicability in bioremediation and biocatalytic systems and to its high tolerance, which could also be a result of the existence of persister cells in the population. Additionally, some strains of *Rhodococcus* have been recently implicated in infections in immunosuppressed patients (*e.g. R. equi*), increasing the interest in studying this species.

The study of these cells must therefore be highlighted as a key issue for the development of more effective and specific therapies for persister-related infections and to avoid their dissemination caused by the widespread use of the available, poorly effective antibiotics and disinfectants. However, before persister-related (chronic) infections could be eradicated, more detailed information regarding the strategies adopted by the bacterial populations to survive a temporary environmental stress is necessary, namely how persisters are formed.

1.1. Persister Cells and Antibiotic Tolerance

Persister cells were first described by Joseph W. Bigger (Bigger, 1944) who studied the action of penicillin against staphylococci. Bigger observed that the addition of the antibiotic to liquid cultures led to the sorting out of a small number of survivors he termed “persisters”. Upon reinoculation, these cells gave rise to a culture that again was susceptible to the antibiotic, forming a new subpopulation of persisters. Decades later, first Lewis (Lewis, 2001) and then Keren *et al.* (Keren *et al.*, 2004a), tested the tolerance of *E. coli* to different antibiotics at distinct growth stages. In both studies, the behavior

observed by Bigger (Bigger, 1944) for staphylococci population susceptibility upon a second antibiotic exposure was obtained, corroborating Bigger's differentiation of persisters from resistant mutants in terms of antibiotic susceptibility (**Figure 1**).

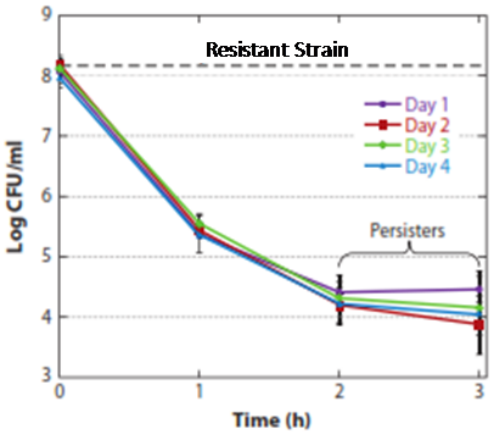

Figure 1 – Population susceptibility upon a second antibiotic exposure. The addition of the antibiotic to an exponentially growing population of *E. coli* at time 0 led to the survival of a small fraction of persister cells. Adapted from (Lewis, 2010).

Furthermore, Bigger concluded that penicillin, as now known to most antibiotics, is not able to eliminate all the bacterial cells contained in a culture, mainly due to persister cell tolerance (**Figure 2**). Detailed information on that subject is given in section ***2.3.2. Resistance versus Persister Tolerance***.

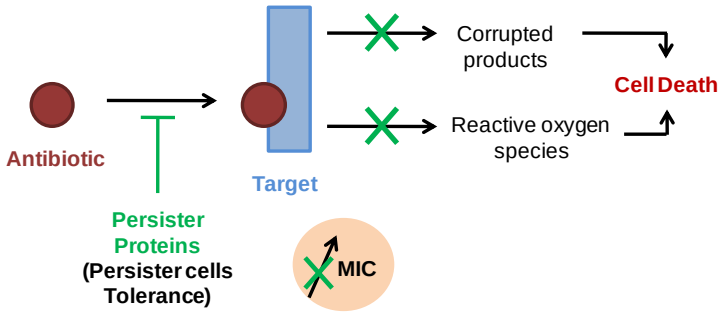

Figure 2 – Bacterial persister cells tolerance. Bactericidal antibiotics kill cells by forcing the (active) target to produce corrupted products (*e.g.*, toxic misfolded peptides). A study performed by Kohanski *et al.* further concluded that bactericidal antibiotics can also induce cell death by triggering the production of reactive oxygen species (Kohanski *et al.*, 2007). Persister cells tolerance is triggered by the so called 'persister proteins' which block the target of the antibiotic, and the subsequent cell death, with no increase in the MIC. Adapted from (Lewis, 2010).

The resistance to bactericidal antibiotics, reported for the first time shortly after health care services began to administer penicillin, and its subsequent spread, quickly started to threaten the efficiency of the (at the time) newly discovered antibiotics. By that time the study of bacterial resistance therefore became a priority, leading to the oblivion of persister cells (Lewis, 2010). These studies, eventually found that although these resistance mechanisms were able to reduce the effectiveness of the existing bactericidal antibiotics (Levy and Marshall, 2004), they did not made them completely useless during the treatment of an infection. These years, from the 1940s through the 1960s, were later termed 'golden era of antibiotics discovery' (Walsh, 2003), during which many effective compounds were produced, thus outpacing the spread of resistance. Doctors and researchers started to think that was the end of the 'war' against bacterial resistance, and less importance was given to research and development of new drugs.

Only forty years later after Bigger's studies, and thanks to a renovated interest of Moyed (and Bertrand) in nonheritable variation, persister cells were placed in a prominent position (Moyed and Bertrand, 1983), although most medical staff and researchers still disregard them. The results obtained in that study led to the identification of a *hipA*7 gain-of-function allele (*hip*, 'high-level persistence') in *E. coli* and for non-mutant cells; any mutants that could grow in the presence of antibiotic (*i.e.*, had a higher MIC) were discarded. However, despite of the promising results, the deletion of that *hipA* allele produced no distinct phenotype (Moyed and Bertrand, 1983), which eventually blurted out its potential role in the formation of persisters. Similarly, although pioneer, the work of Moyed and Bertrand, and once more the persisters, were forgotten, this time for a ten-year period. With the 1990s came the fast spreading of resistance and the rise of chronic infections (apart from the acute infections), surpassing the discovery of new, more effective antibiotics. At the time, the widespread use of indwelling devices and the increase in immunocompromised patients due to cancer chemotherapy and HIV led to high fatality rates, with many chronic infections found to be associated to bacterial biofilms (Costerton *et al.*, 1999). A few years later, and already in the twentieth century, a study of dose-dependent killing of a *P. aeruginosa* biofilm, performed by Spoering and Lewis, showed the presence of a small subpopulation of cells completely tolerant to antibiotics (Spoering and Lewis, 2001). Persister cells, this time within biofilms, were pointed to the likely culprit of antibiotic recalcitrance. Once more, the study of persisters gained a renewed interest, which endures to the present day. However, and although it has been showed that persisters are likely to be the main cause for the recalcitrance of chronic infections to antimicrobials (Lewis, 2010), establishing causality and link of persisters to diseases is yet, sometimes, a problem.

1.2. Purpose and Objectives of This Thesis

Despite their discovery almost 70 years ago, a disparity between the significance of the problem of persister cells and the modest pace of progress in the field still exists, mainly due to a combination of two factors: i) uncertain significance; and ii) redundancy of mechanisms (Lewis, 2010). The findings

linking persisters to chronic infections such as tuberculosis and oral thrush support what has been suggested: persisters are responsible for the recalcitrance of chronic infections (Keren *et al.*, 2004a; Moyed and Bertrand, 1983; Spoering and Lewis, 2001). The other big remaining questions are the mechanism(s) of persister formation and how to accomplish persister eradication. Further studies will be of paramount importance to provide more detailed information.

The main objectives of the master studies proposed were thus the following:

- to study the appearance of persister cells in antibiotic exposed populations;
- to study the effect of antibiotics on the fatty acid composition of the phospholipids of persister bacterial cells;
- to study their effect on the surface properties of the cells.

2. BACKGROUND

2.1. The Rise of the Chronic Infection Era

Bacteria that attach to surfaces may aggregate in a hydrated polymeric matrix of their own synthesis, known as biofilm. Recalcitrant diseases, like tuberculosis, are accompanied by the formation of these structures, which may be a source for continuous infection. Such infections have a slower progression than acute infections and their symptoms are often vague (often referred to as 'low grade') (Costerton, 2007). The classic examples, prior to the wide application of antibiotics, included tuberculosis and leprosy, both slowly degrading the tissue and affecting organs of patients, and eventually leading to death.

Chronic infections usually establish and develop in patients who suffer from diseases or conditions that cause deficiencies in the primary defensive barriers (innate immunity). This includes disruption of the anatomical (*e.g.*, skin, mucous membranes and cilia) and physiological (*e.g.*, temperature and low pH) inflammatory barriers, as well as phagocytic defects (*e.g.*, macrophages) (Bjarnsholt, 2013). Bjarnsholt divided these deficiencies into 3 categories: 1) congenital abnormalities; 2) the presence of foreign bodies; and 3) acquired chronic diseases. The classic example of the former is the chronic infection found in patients suffering from cystic fibrosis (CF) (Høiby, 1977). The presence of foreign bodies such as artificial limbs, cardiac valves and indwelling catheters present an adhesion surface to bacteria and a site entrance to the body, while injected tissue fillers are also now being reported as a site of chronic infections (Bjarnsholt, 2009). Finally, patients suffering from acquired chronic diseases may be prone to the development of a non-healing wound that is chronically infected with bacteria (*e.g.*, diabetes mellitus), or to chronic lung infections (*e.g.*, COPD) (Brusselle, 2011).

2.2. Persistence Evidence in Biofilms

Since the development of antibiotics, the number of chronic infections has been increasing compared to those of acute infections (Costerton *et al.*, 1999). A series of *in* vivo and *in vitro* observations made by Costerton at the final of the last decade (Costerton, 2007), identified the sources of these persistent infections. These observations accounted the capacity of bacteria to aggregate as the cause of slow-progressing infections.

A biofilm has been defined as a population of cells growing on a surface (or not (Bjarnsholt, 2013)) and enclosed in an extracellular matrix that contains many different types of extracellular polymeric substances (EPS) including proteins, DNA, lipids and polysaccharides. Cells establish this form of self-immobilization when they adhere to surfaces in contact with water (Costerton et al., 1999; de Carvalho and de Fonseca, 2007a). According to the *National Institutes of Health (NIH)*, *U. S. Department of Health & Human Services* (http://www.nih.gov/), "more than 60% of all microbial infections are caused by biofilms". The important role of biofilms in these microbial infections is explained by the fact that, the additional protection that biofilms give to the cell fraction contained within them during exposure to a given drug provide an important reservoir of cells that can repopulate and colonize sites upon reduction or removal of the drug levels. Since the cells in these aggregates can withstand doses of antibiotics that would kill planktonic cells, biofilms are notoriously difficult to eradicate.

The high tolerance observed in biofilm cells results from the fact that bacterial cells (even the usually regular, susceptible majority of cells) are much more protected from antimicrobial agents when embedded in biofilms than planktonic cells thanks to a combination of mechanisms, *e.g.* reduced diffusion due to the exopolymeric matrix, physiological changes in the cells due to reduced growth rates, and production of enzymes able to degrade the antimicrobial substances (Stewart and Costerton, 2001). Therefore, biofilm tolerance does not depend only on the presence of persister cells, but also on strictly molecular mechanisms. Biofilm antibiotic tolerance should not, however, be confused with antibiotic resistance. Resistance is an ability of a microorganism to grow in the presence of an elevated level of a given antimicrobial agent due to an increase in the initial (observed previously to exposure) MIC. By this conventional criterion, biofilm cells do not necessarily show increased resistance (Lewis, 2001). Tolerance against host defenses is also dramatically increased in biofilm-contained cells (Bjarnsholt, 2013), for the same protection principles described above for antimicrobial agents. Importantly, although many chronic infections involve surfaces (*e.g.*, infections on implants, catheters, or contact lenses), more recent observations of non-surface-related infections (*e.g.*, otitis media or chronic wounds), have been found to use the same patterns (Bjarnsholt, 2013). Furthermore, these persister-related infections (*e.g.*, tuberculosis) are in most cases accompanied by the formation of biofilms, which can be formed by the majority of the pathogens (Keren *et al.*, 2004a; Moyed and Bertrand, 1983; Spoering and Lewis, 2001). This capacity of pathogens to form such a structure, which presents a barrier that protects the cells therein, thus seems to indicate the source of continuous infections (Costerton *et al.*, 1999).

Biofilm infections affect millions of people all around the world each year and many deaths occur as a consequence of the presence of such a structure. The *World Health Organization (WHO)* recently reported (*Cases: Mortality and prevalence by WHO region (2007 - present),* http://apps.who.int/gho/data/view.main.57016ALL?lang=en; accessed January 2014) that in 2011 between 990,000 and 1,100,000 deaths resulted directly from tuberculosis (excluding HIV). More particularly, in Europe an average of 45,000 deaths were accounted during the same period; the worst scenario was observed in South-East Asia, with an average of 480,000 deaths. These high numbers reflect the reduced results of the current, low successful treatment of human tuberculosis, which requires 6-9 months of therapy with multiple antibiotics (Ojha *et al.*, 2008). Incomplete clearance of the bacilli frequently results in disease relapse, presumably as a result of reactivation of persistent drug-tolerant *Mycobacterium tuberculosis* cells, although the nature and location of these persisters are not known (Ojha *et al.*, 2008).

2.3. Bacterial Resistance and Persistence

Persisters are highly antibiotic tolerant variants of regular bacterial cells that form stochastically in microbial populations (Lewis, 2010). This fraction of cells enters in a multidrug-tolerant state, thus, neither grows nor dies in the presence of microbial antibiotics (and other antimicrobial agents). But how do persister cells, unlike regular, susceptible cells, manage to survive exposure to an antibiotic? Most of the Antibiotics do not actually kill cells but rather cause damage to them. Cells with serious defects will then, eventually, undergo programmed cell death (PCD) (Lewis, 2001). Identical behaviors are observed in animal cells, whose death results, in most cases, from apoptosis induced by damage from toxic factors (Lewis, 2001). Especially for a bacterial population, this strategy involving the 'suicide' of defective cells, which would otherwise consume the so needed, and sometimes scarce, resources of the healthy, neighbor cells, may reveal to be of great importance in terms of adaptation. The elimination of all cells upon antibiotic addition would be, however, counterproductive for the bacterial population. Once more, persister cells are the ones responsible for the prevention of such an occurrence, by showing a disabled PCD. Though effective, this mechanism of survival must be meticulously controlled. Importantly, cells would need to discriminate between an unrepairable defect and temporary starvation conditions.

When exposed to an antibiotic, bacterial populations follow a biphasic pattern, with a distinct plateau of surviving persisters (Lewis, 2010). According to Lechner *et al.* (Lechner *et al.*, 2012), persister tolerance is not transferred to the progeny, therefore, a similar behavior to that observed during the first exposure is expected upon successive antibiotic additions of the previous survival persister subpopulation. On the other hand, resistance mechanisms, which confer to cells the ability to grow in the presence of (elevated concentration of) antibiotics by preventing these chemical compounds from binding to their targets, and thus increasing cells MIC, are genetically transferred to the progeny (Lewis, 2001). Therefore, opposing to the biphasic killing pattern observed for the re-grown population

from the small fraction of survival persisters, resistant, mutant cells show, upon successive additions of a given antibiotic an ability to tolerate increased concentrations of antibiotics (Lewis, 2010).

2.3.1. The Role of Persister cells in Chronic Infections

Chronic infections, often accompanied by the formation of biofilms, are usually recalcitrant to antibiotic treatment, even when caused by a pathogen that is not resistant to that specific antibiotic. *Pseudomonas aeruginosa*, which may cause incurable infection in CF patients, and *S. aureus* and *S. epidermidis*, which cause mainly infections of indwelling devices, are probably the best-studied biofilm-producing organisms (Lewis, 2007). However, planktonic cells that are derived from these biofilms are, in most cases, fully susceptible to antibiotics (Lewis, 2007). Importantly, biofilms do not actually grow in the presence of elevated concentrations of antibiotics, therefore biofilms do not have increased resistance compared to planktonic cells. This means that these cells must have special mechanisms that allow them to survive antibiotic exposure others than the ones conferring resistance. The resistance of biofilms to antibiotics has been one of the more subtle problems in microbiology, but the analysis of a dose-response experiment of *P. aeruginosa* biofilms to the quinolones ofloxacin and ciprofloxacin provided an unexpected insight into the 'puzzle': similarly to what is observed with planktonic cells, low concentrations of antibiotics effectively eliminate the majority of biofilm-contained cells (Brooun et al., 2000); thus suggesting the presence of a tolerant subpopulation of persister cells within biofilms. Further studies showed that most of the cells in a biofilm are actually highly susceptible to bactericidal agents such as fluoroquinolone antibiotics or metal oxyanions, which can kill both rapidly dividing and slow- or non-growing cells (Harrison *et al.*, 2005; Spoering and Lewis, 2001). This is important, since cells in the biofilm are indeed slow-growing cells, and many are probably even in stationary growth phase. The study presented by Lewis also revealed a small sub-population of cells – persister cells – that remained alive irrespective of the concentration of the antibiotic added (Lewis, 2001). Furthermore, the number of surviving persisters was greater in the non-growing stationary phase. According to the author's *in vitro* results, a stationary culture seems to be more tolerant than a biofilm to antibiotics. However, this situation is probably reversed *in vivo*. On the basis of these findings, the author proposed a simple model of a relapsing chronic infection: an antibiotic addition will kill most biofilm and planktonic cells, leaving persisters alive. At this point, the similarity with an *in vitro* experiment probably ends. Next, the immune system can kill the remaining planktonic persisters, just as it eliminates non-growing cells of a bacterial population that is exposed to a bacteriostatic antibiotic. However, and here is where biofilms tolerance becomes interesting, the biofilm EPM protects against immune cells by forming a barrier for its larger components (Lewis, 2010). Thanks to that capacity, the presence of biofilm-specific resistance mechanisms was suggested in the last decade to account for the recalcitrance of infectious diseases (Stewart and Costerton, 2001). That way, persisters that are contained in the biofilm can survive both the addition of antibiotics and the action of the immune system of the host (**Figure 3**). Afterwards, when the concentration of antibiotic is reduced, the persisters can repopulate the biofilm, which will produce new planktonic cells, originating the relapsing

of biofilm infection (Lewis, 2001). However, the problem of biofilm resistance to killing by most therapeutics is most likely due mainly to the presence of persisters rather than to simple biofilm matrices. Alternatively, another model was later hypothesized by Lewis (Lewis, 2010): rather than persisters repopulation of the biofilm once the concentration of antibiotic reduces, this new model simply states that antibiotics might fail to effectively reach at least some cells *in vivo*, resulting in a relapsing infection.

Interestingly, yeast biofilms also form tolerant persisters. These persister cells, in common with bacterial biofilms, are responsible for highly recalcitrant infections caused by yeasts (Kumamoto and Vinces, 2005). Together with LaFleur and one of the authors of the paper that revealed the presence of these persisters in yeasts, Lewis performed a dose-dependent experiment with two microbicidal agents, amphotericin B and chlorhexidine, in order to examine the biofilm resistance of *Candida albicans* (LaFleur *et al.*, 2006), following the same approaches that were previously used for bacteria. Although a complete elimination of cells in exponential and stationary planktonic populations were observed, biphasic killing was also registered in a mature biofilm, therefore indicating the presence of persisters (LaFleur *et al.*, 2006). Again in common with bacteria, yeast persisters are not mutants, since upon their reinoculation the population followed the same normal behavior, with surviving cells showing the same features as the original wild-type population, with a new fraction of persister cells produced. Moreover, the authors further decided to analyze cell viability by fluorescence microscopy and image analysis, using fluorescein acetate, which specifically binds to dead yeast cells. The results showed live persisters in a yeast biofilm after exposure to amphotericin B, which were either yeast or pseudo-hyphal forms; they were morphologically unremarkable. Furthermore, sorting of this population showed that dim cells were able to form colonies, whereas bright ones were not. Importantly, and in contrast with bacteria, *C. albicans* persisters were only found under the form of biofilm-grown cells, and were not present in a non-growing, stationary phase population (LaFleur *et al.*, 2006); recall that in bacterial populations a stationary planktonic culture contains more persisters than a biofilm (Spoering and Lewis, 2001).

Even so, establishing causality between persisters and therapy failure is not trivial, because these cells form a small subpopulation with a temporary phenotype, which precludes introducing them into an animal model of infection. According to Moyed and Bertrand, periodic application of high doses of bactericidal antibiotics leads to the selection of strains that produce increased levels of persisters (Moyed and Bertrand, 1983), therefore the causality can be tested based on what is known about *in vitro* selection of high persistent *hips* mutants. In fact, this is exactly what happens during a chronic infections treatment. However, *hips* would only gain advantage if the drugs effectively reach and kill the regular cells of the pathogen, and unfortunately that is not always the case. Considering the classical example of CF: patients are usually treated for decades for an incurable infection (caused by a number of pathogens, like *P. aeruginosa*), to which they eventually succumb. The periodic application of high doses of antibiotics provides CF patients some relief by reducing, although temporary, the number of pathogenic cells; it does not clear the infection. If *hip* strains of pathogens were selected *in vivo*, they would most likely be present in a CF patient, as recently demonstrated (Mulcahy *et al.*, 2010). Testing persister levels by monitoring the survival of a set of longitudinal *P.*

aeruginosa isolates (collected from a single CF patient over the course of several years) after challenge with a high dose of ofloxacin showed a dramatic, 100-fold increase in surviving cells in the last four isolates (Mulcahy *et al.*, 2010). Furthermore, testing paired strains from additional patients showed, in most cases, a considerable increase in persister levels collected in the patient throughout the progression of the disease (Mulcahy *et al.*, 2010). Importantly, most of the *hip* isolates suffered no increase in MIC compared with their clonal parent strain upon exposure to different antibiotics (Mulcahy *et al.*, 2010), suggesting that classical acquired resistance plays little to no role in the recalcitrance of CF infection. These experiments directly linked persisters to the clinical manifestation of CF infections and suggested that persisters are responsible for the therapy failure of this, and most likely other, chronic infections.

Interestingly in chronic infections caused simultaneously, by different species (*e.g.*, *P. aeruginosa* and *C. albicans*), and given the similar lifestyles of these unrelated microorganisms, it is likely that the advantage that the bacteria gets from the survival of a *hip* is universal. Similarly, and considering as an example the yeast *C. albicans*, the production of persisters occurs through mechanisms that are probably analogous (LaFleur *et al.*, 2006), rather than homologous, to the ones used by their bacterial counterparts.

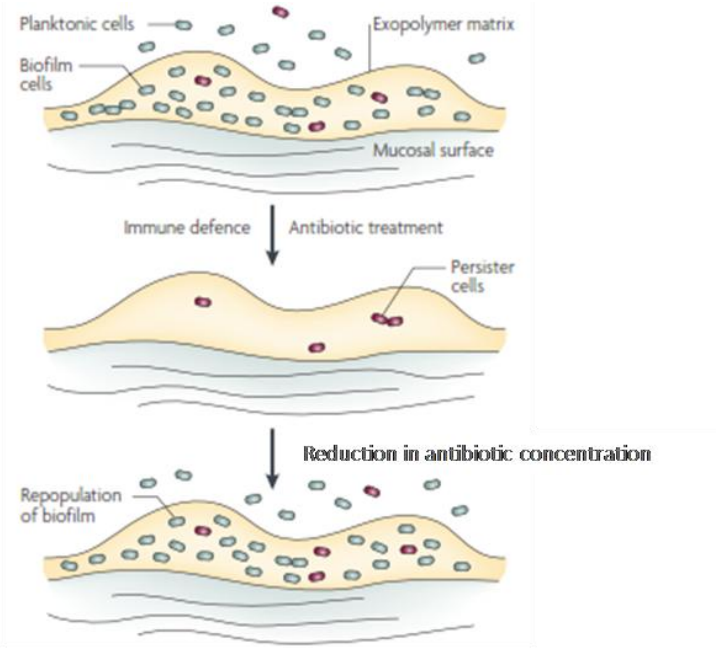

Figure 3 – A simple model of a relapsing chronic infection. A model of biofilm resistance to killing based on persister survival is presented. The initial antibiotic exposure kills normal cells (cells colored green) in both planktonic and biofilm populations, leaving persisters in both populations alive (cells colored pink). Next, the immune system kills the remaining planktonic persisters (cells colored pink outside the biofilm), but not the biofilm persister cells (cells colored pink within the biofilm), which are protected from the host defenses by the EPM. After the antibiotic concentration is reduced, persisters allow the re-population of the biofilm, and the infection relapses. Adapted from (Lewis, 2007).

In 2010, Lewis introduced a new, more general paradigm on the role of persisters in chronic infections (Lewis, 2010). In that publication, Lewis stated that “persisters are critical for pathogens to survive antimicrobial chemotherapy whenever the immune response is limited”, conditions included cases of disseminating infections in immunocompromised patients undergoing cancer chemotherapy or infected with HIV. Cases of immunocompetent individuals where the pathogen is located at sites poorly accessible by components of the immune system are included as well, like the central nervous system, in which pathogens cause debilitating meningitis and brain abscesses, and the gastrointestinal tract, where the hard-to-eradicate *Helicobacter pylori* causes gastroduodenal ulcers and gastric carcinoma (Lewis, 2010).

Tuberculosis, caused by *Mycobacterium tuberculosis*, is one of the most prominent cases of a chronic infection caused by a pathogen evading the immune system (Fox, 1999). For the survival of infected patients, there are two potential scenarios: i) the acute infection may resolve by itself; or ii) as a result of antimicrobial therapy. In the later case, the therapy usually comprises the use of three antibiotics, isoniazid, rifampicin and pyrazinamide, for a period of at least six months. For years, such intensive therapy was thought to kill the overall pathogen population. However, it is now known that this is not the case, with a small subpopulation actually becoming highly antibiotic-tolerant, through phenotypic modifications (Jindani et al., 2003; Saltini, 2006). Although the pathogen often remains in a ‘latent’ form, that actually serves as the main reservoir of tuberculosis infection (Barry *et al.*, 2009), there are still no conclusive results confirming that the persisters in latent infection are exclusively in a non-replicative state (Gomez and McKinney, 2003; Parrish et al., 1998). In fact, Gill and co-workers reported an active replication for *M. tuberculosis* bacilli during the chronic phase of infection in a mouse model (Gill et al., 2009). These results strongly suggest the presence of active rather than ‘dormant’ persister cells during a phase of both absence of symptoms of the disease by the host and stable number of live bacterial cells. Equivalent results were later obtained by Ford and co-workers (Ford et al., 2011). These authors observed a similar rate of accumulation of mutations in *M. tuberculosis* populations for either latent or active infections in non-human primates. Moreover, both were similar to a logarithmically growing *in vitro* culture, suggesting active DNA replication and subsequent cell division of the pathogen in latent infection. Nevertheless, further studies are needed before any conclusions may be driven from those observations.

2.3.2. Resistance versus Persister Tolerance

By monitoring the growth curves of a challenged population, it is possible to access tolerance and resistance of the cells. CFU counts over time from a culture exposed to an antibiotic produces a biphasic pattern with a distinct plateau of surviving persisters (**Figure 4**). On the other hand, resistant cells are expected to grow in the presence of the antibiotic.

Since the mechanisms of resistance prevent antibiotics from binding to their targets by altering them in a way that they fail to bind it, it is possible to quantify the level of resistance by observing the ability of cells to grow in the presence of (increasing concentrations of) antibiotics, *i.e.*, by looking for the lowest

concentration of the antibiotic allowing no growth (≡ MIC). For that, the concentration of a given antibiotic is serially diluted in a microtiter plate containing cells and growth medium and, after a period of incubation, the MIC is evaluated. The main types of resistance are target modification by mutation; target modification by specialized enzymatic changes; target substitution, such as expressing an alternative target; antibiotic modification or destruction; antibiotic efflux; and restricted permeability to antibiotics (Lewis, 2007). Importantly, all these mechanisms have, and fulfill, the same final objective which is to prevent the antibiotic from binding to its target in such a way that neither corrupted products nor reactive oxygen species are produced (**Figure 5**) and, therefore, allow cells to grow at an increased concentration of antibiotic. As a result, resistant, mutant cells exhibit an increased MIC.

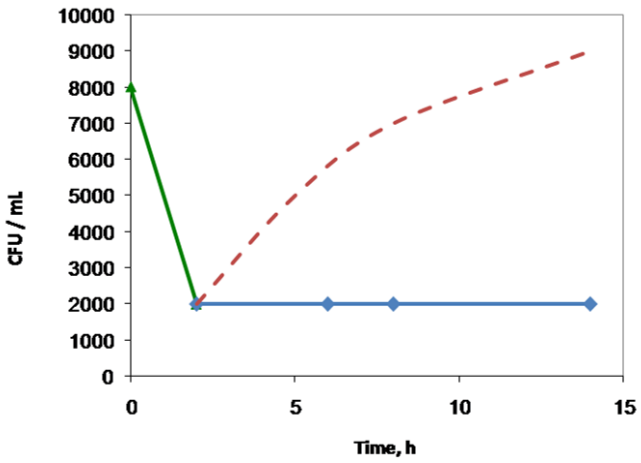

Figure 4 – Biphasic killing pattern. The curves show the evolution of CFU/mL during the exposure of a population (green solid line) to an antibiotic. The result is a biphasic cell death pattern, with a distinct plateau of surviving persisters (blue solid line) or resistant, mutants cells (red dotted line). Adapted from (Lewis, 2007).

Aminoglycoside antibiotics, for instance, kill the cell by interrupting translation, which produces misfolded toxic peptides (Davis *et al.*, 1986). Another group of antibiotics, the β-lactams, such as penicillin, kill the cell through the action of autolysin enzymes present in the cell wall, which are activated (by a yet unknown mechanism) as a result of peptidoglycan synthesis inhibition. Fluoroquinolones inhibit the ligase step of the DNA gyrase and topoisomerase, without affecting the preceding nicking activity. As a result, the enzymes are converted into endonucleases (Hooper, 2002). Glycopeptides, such as vancomycin (**Figure A. 1**) and teicoplanin (**Figure A. 2**), interfere with the late stages of cell wall biosynthesis (Bambeke, 2004; Cazzola et al., 2004)). These two potent glycopeptides form a stoechiometric complex with the D-Ala-D-Ala dipeptide via the formation of five hydrogen bonds with the peptidic backbone, preventing both the transglycosylation and

transpeptidation reactions and thus the incorporation of the precursors into the rigid bacterial cell wall peptidoglycan (Allen et al., 1996; Bambeke, 2004; Nagarajan, 1991).

In contrast to resistance, persister tolerance to antibiotics seems likely to function by the action of 'persister proteins', which prevent the corruption of the cell by a bactericidal agent through the blocking of the antibiotic target(s) (**Figure 5**). If persisters exhibit a dormant-state phenotype (*e.g.* little or no cell wall synthesis, translation or topoisomerase activity), the antibiotics will bind to, but will be unable to corrupt the function of their target molecules (Lewis, 2007), since they need an active target in order to act and cause cell death.

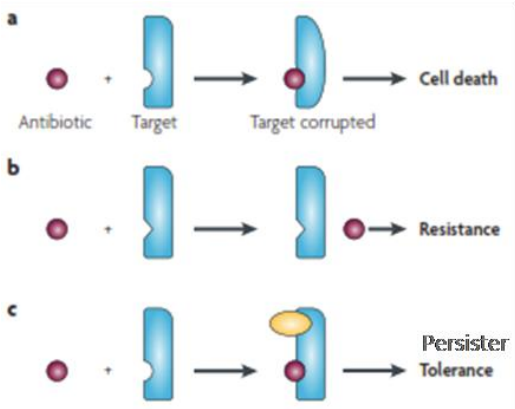

Figure 5 a) to c) – Resistance versus tolerance to bactericidal antibiotics. **a)** The antibiotic (colored pink) binds to the target (colored blue) altering its function and causing cell death. **b)** The target of the antibiotic has been altered so that it fails to bind the antibiotic and the cell becomes resistant to addition of the drug. **c)** A different molecule (colored yellow) inhibits the antibiotic target. This prevents the antibiotic from corrupting its functions, resulting in persister tolerance. Adapted from (Lewis, 2007).

2.3.3. Processes to Access Persister Formation

As reviewed by Lewis (Lewis, 2010), probably the most utilized technique in order to study and find new mechanisms associated to a complex function is by screening knockout libraries in search for specific genes that are already known to be, or that are seen as candidate genes to be involved in that specific mechanism. Among the highly specific, complex knockout libraries, screening a library of transposon (Tn) insertion mutants is seen as one of the most straightforward approaches to follow (Lewis, 2010). By performing this approach, a set of candidate genes should be produced. The subsequent analysis of these preliminary outputs leads to a pathway and, hopefully, to a particular mechanism (Spoering *et al.*, 2006). Although many basic mechanisms have already been established by this type of approaches (*e.g.*, mechanisms of sporulation, flagellation, chemotaxis, virulence) (Lewis, 2010), not always the obtained results are conclusive. Screening a Tn insertion library of *E. coli* for an ability to tolerate high doses of antibiotics, produced no mutants completely lacking

persisters (Hu and Coates, 2005; Spoering *et al.*, 2006), therefore precluding the identification of the genes required for persister formation. These results led Spoering *et al.*, as well as all the other research groups in the field whose results were also unclear, to rethink the standard 'one phenotype/one mechanism' model usually accepted. Given the absence of detailed mechanistic knowledge on persistence due to these inconclusive, genetic results, researchers shifted the approaches followed towards other reliable, direct methods of studying the evolution of cells. As recently revisited by Patra (Patra and Klumpp, 2013), 'population dynamics' studied upon antibiotic exposure, in particular, the survival upon administration of the drug and the regrowth of the population upon removal of the drug, revealed to be an effective approach to gain insight on the nature of persister cells. These techniques are based on the analysis of the rate of persister-cell formation over time. Using them, Keren *et al.* examined the evolution of several species, including *E. coli*, *P. aeruginosa* and *S. aureus* (Keren *et al.*, 2004a). All species presented similar dynamics, suggesting that few persisters are formed in early exponential phase, followed by a sharp increase in persister-cell formation during mid-exponential phase, and a maximum of *ca.* 1% of cells were persisters during the non-growing stationary phase (Keren *et al.*, 2004a). This shows that the production of persisters depends on the growth stage/culture age (**Figure 6**).

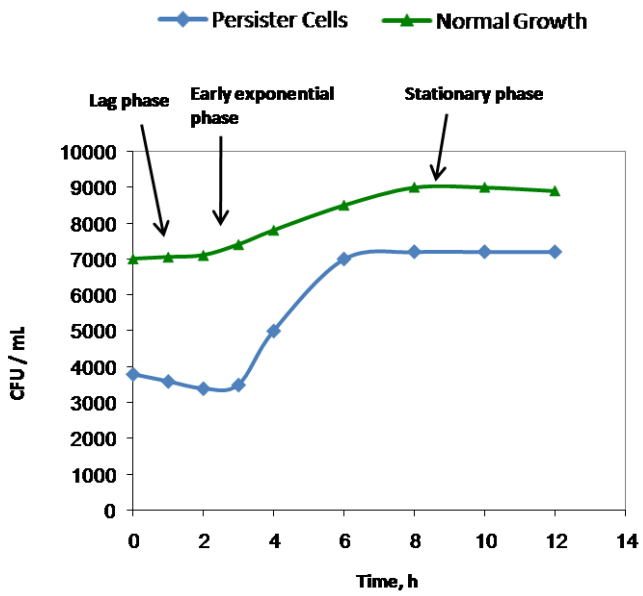

Figure 6 – Dependence of persister-cell formation on growth stage. The curves depict a bacterial normal growth (green line) and the frequency of isolation of persister cells (blue line) as a function of the growth phase of the culture. Adapted from (Lewis, 2007).

Maintenance of a population in early exponential state by serial reinoculation resulted in complete disappearance of persisters after as few as four cycles (Keren *et al.*, 2004a). These results strongly suggest that the persisters isolated during the early exponential phase of growth were introduced from

a stationary state culture inoculum (carryover effect), rather than formed *de novo* in response to antibiotics. Therefore, it was proposed that no persisters are formed during early exponential growth phase (Keren *et al.*, 2004a). Keren's study on the nature of persisters also ruled out non-specific mechanisms of persister formation. That said persisters, although exhibiting a genetically identical kin in a given population which would suggest a stochastic formation process (Keren *et al.*, 2004b), the dramatic increase in persister levels from mid-exponential to stationary phases of growth in all species tested so far (Keren *et al.*, 2004a; Spoering and Lewis, 2001) indicates that there is also a deterministic component controlling persister formation, otherwise persister-cells formation would be expected to occur throughout the growth of the culture (Keren *et al.*, 2004a). However, and quite interestingly, only by 'noisy', stochasticity processes it is possible to create a 'useful variety' within a population, contradicting the well-established evolutionary paradigm of evolving to perfection by economy-driven regulatory functions (Lewis, 2007). Therefore, the mechanism that underlies the sharp increase of persister cells formation during mid-exponential phase still remains unknown. Quorum sensing was hypothesized to have a role in persister-cell formation (Bassler and Losick, 2006), however, the followed mechanism does not fit that theory, since the addition of spent growth medium (which would contain high concentrations of quorum-sensing signaling molecules) to early exponential cultures of *E. coli* or *P. aeruginosa* did not appreciably increased the number of persisters isolated (Lewis, 2010).

Regardless of the mechanism, the dynamics of persister formation provide an interesting insight into the process. Moreover, since the highest frequency of persister formation occurs during the stationary growth phase, the main function of this class of persister cells might be to ensure the survival of the non-growing population. As persisters do not grow, it would seem logical for the entire stationary population to enter into this protected survival state, but this is not the case. The benefit of being a regular, dividing cell seems to be attributed to its ability to rapidly resume growth, a process that takes 1-1.5 hours longer for an 'inactive', persister cell (Balaban *et al.*, 2004). Most cells in an *E. coli* or *P. aeruginosa* stationary phase population are in fact non-persisters, which indicates that, and opposed to what would be expected, the optimal individual strategy is not to enter into persistence. This led to the conclusion that the persister state is an 'altruistic' behavior benefiting the kin (Lewis, 2007). Indeed, in the rare instance that nearly the whole population of normal cells is killed by a lethal factor, the surviving persisters can propagate the genome that they share with their kin.

A similar behavior can be found in unculturable/unfamiliar environment-exposed bacteria (Lewis, 2007). Lewis observed that organisms which are unculturable alone will readily grow on nutrient medium on a Petri dish *in vitro* in the presence of other species collected from the same environment. This results might suggest that growth depends on substances (*e.g.*, signaling molecules) that indicate the presence of a familiar, enriched in essential molecules (which the unculturable species are not able to synthesize by themselves) environment. Therefore, a cell dividing in an unfamiliar, but nutrient-rich environment exposes itself to an uncertain fate of being killed by an unfamiliar antibiotic to which it has no resistance. Given that even a simple lack of growth is a partial state of 'inactivity' that might confer protective advantages to the population (Lewis, 2007), Lewis hypothesized that when exposed

to an unfamiliar environment unculturable cells enter into a dormant state that is similar to persisters 'inactive' state experienced upon, *e.g.*, antibiotic exposure.

Another curious fact regarding persister formation was observed by Wiuff and co-workers: in populations grown under seemingly identical conditions but challenged with different antibiotics, although typical biphasic killing kinetics were observed for each tested antibiotic, the different killing curves trajectories obtained indicate physiological heterogeneity in terms of robustness or fitness within persister subpopulations (Wiuff et al., 2005). Since variability of cells within a population increases the chances of kin survival (Lewis, 2010), this observed variability within persister subpopulations, though not fully understand yet, will likely be similarly adaptive.

2.3.4. Persisters – 'Dormant' or Active Cells?

Whether persister cells are in fact in a dormant, inactive state or, instead, that the persistent phenotype corresponds actually to an active response of cells to stress agents remains yet unclear. Since persisters were first described by Bigger, 70 years ago (Bigger, 1944) their associated capacity to tolerate high antibiotic concentrations have been generally attributed to phenotypically reversible modifications and subsequent entrance in a 'dormant', slowly- or non-dividing state. However, recent evidences have been supporting the exact opposite. Earlier in 2013, Wakamoto, Dhar and colleagues observed, for antibiotic-exposed *Mycobacterium smegmatis*, that rather than enter a dormant state, persister cells actually enter a "dynamic state of balanced cell division and death" upon antibiotic exposure (Wakamoto et al., 2013). In the same line of reasoning, and with just a six-month interval, Orman and Brynildsen designed an experiment to differentiate, in *E. coli*, non-dormant, actively dividing and metabolically active cells from dormant cells (Orman and Brynildsen, 2013). By performing a fluorescence activated cell sorting (FACS), the authors observed that, although they are more likely to correspond to non-growing cells than to growing cells, persisters can also be found in the normally growing subpopulation. Furthermore, the authors obtained a set of interestingly results: a large fraction of the actively growing cells were metabolically inactive or dormant and, in parallel, a large fraction of the non-growing cells were metabolically active. That way, the authors concluded that the dormancy is neither necessary nor sufficient for persistence. In the same year, and following the work of Johnson and Levin (Johnson and Levin, 2013), who used mathematical models and experimented in *S. aureus* exposed to a series of different antibiotics to propose that there are different types of persister cells formed by a variety of mechanisms, Hofsteenge *et al.* observed different tolerances to different antibiotics (and even for antibiotics with nearly identical mechanisms of action) in non-pathogenic *E. coli* environmental isolates (Hofsteenge et al., 2013). Those results led the author to conclude the existence of "distinct physiological states of dormancy, each of which is differently susceptible to a particular antibiotic". Based on their observations, and according to the results of Johnson and Levin, the authors hypothesized that not only the dormancy, but also additional physiological changes are responsible for persister phenotypes. Given the most recent advances on this hot topic, and due to the latest evidences that persister cells are not dormant but rather in an

active state, which allows cells to respond to stress agents, it seems now more than before that a consensus can be reached.

2.4. The Search for 'Persister Genes'

As already mentioned in the previous section, attempts to identify genes that are required for persister-cell formation – 'persister genes' – have been unsuccessful (Hu and Coates, 2005; Spoering et al., 2006). The main reason for these failed efforts is the variation in the numbers of persisters identified in parallel replicates (Wiuff et al., 2005), which adulterates the results obtained when screening a large library, by creating high levels of both false-positives and false-negatives. Another genetic method, in alternative to screening a knockout library, is to identify genes by gain-of-function from an expression library. However, this technique has its own problems. The overproduction of some proteins, *e.g.*, can result in toxic effects that restrict cell growth and thus, once more, originate false-positive results (Lewis, 2007). Unfortunately, this second genetic method also proved inefficient in the identification of persister genes. Despite the urgent need for techniques to identify persister genes, the available standard genetic approaches demanded further advances before conclusive results could be obtained. The development of the complete, ordered *E. coli* gene knockout library, by the Mori group (the Keio collection), was one of these promising advances, since it were never discarded the possibilities that the previous attempts might have failed also by the missing of a critical gene by the transposons or to the small length of the library (Baba et al., 2006). The screening approach was then revisited using the Keio collection (Hansen et al., 2008), but again, no mutants lacking persisters were observed, suggesting a high degree of redundancy in the mechanisms of persister formation. A number of genes, mainly global regulators (*e.g.*, *DksA*, *DnaKJ*, *HupAB*, and *IhfAB*) were, however, identified whose knockouts showed a *ca.* 10-fold decrease in persister formation (Hansen et al., 2008). These results therefore further indicate a real degree of redundancy in persister-cells formation mechanisms, in which a global regulator can affect, simultaneously, the expression of several persister genes, producing the final persister phenotype (**Figure 7**). Two additional genes, *YgfA* and *YigB*, were also identified in the screen which may have a more direct role in persister formation itself. The former is known to be able to inhibit nucleotide synthesis; the last is able to block metabolism by depleting the pool of flavin mononucleotide (Hansen et al., 2008). More recently, an equivalent screen was also performed in *P. aeruginosa* (De Groote et al., 2009). Similarly to what was observed for *E. coli*, no mutants completely lacking persisters were obtained, confirming the existence of such redundant mechanisms of persister formation in other bacteria.

The studies performed so far, clearly indicate that persister-cells formation, unlike complex cellular functions which have a single linear regulatory pathway controlling an execution mechanism, may progress through a number of independent parallel mechanisms (**Figure 7**). Thanks to this bacterial redundant strategy, no single compound should be able to completely inhibit persister formation. Redundant pathways provide a great adaptive advantage to the bacterial population, precluding both natural and synthetic production of compounds that will succeed in binding simultaneously to different targets.

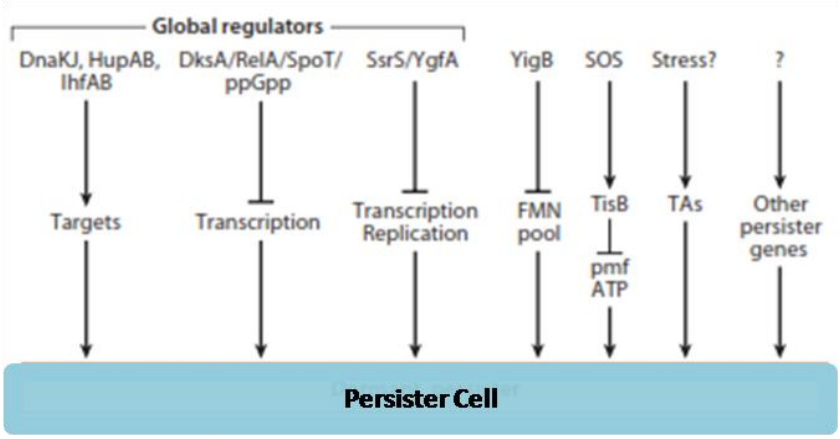

Figure 7 – Redundant pathways of persister formation. Candidate persister genes and their targets, when known, are indicated. Abbreviations: FMN, flavin mononucleotide; pmf, proton motive force; TA, toxin/antitoxin modules. Adapted from (Lewis, 2010).

Screening approaches performed to date in an attempt to identify persister genes were useful only in finding some possible candidate genes (Hansen et al., 2008) besides pointing to redundancy of function (De Groote et al., 2009; Hansen et al., 2008). Transcriptome analysis could be an approach to identify persister genes. For that kind of analysis, however, the persisters have to be isolated first. Given that persisters form a small and temporary population, isolation can be challenging. Therefore, another barrier to the identification of persister genes has been the lack of methods for the isolation of persister cells. According to Keren *et al.* (Keren et al., 2004b), a sufficient amount of persisters needed to perform a transcriptome analysis can be isolated from a bacterial population of growing cells by the addition of a β-lactam antibiotic to the culture followed by the collection of the fraction of surviving persisters. However, in the end, although useful, the transcriptome resultant from such an analysis is neither entirely reliable nor accurate (Lewis, 2010). Following the study of Keren *et al.* on persister isolation, a more advanced method was developed. Shah *et al.* (Shah et al., 2006) reported that if a strain was manipulated in order to express degradable GFP, the non- or slow-growing, persister cells will become dim (**Figure 8**). This pattern was, in fact, observed for a population of *E. coli* after such a manipulation (Shah et al., 2006) (**Figure 8**). Using degradable GFP expression, the difference in fluorescence allowed the two subpopulations to be sorted (Shah et al., 2006). Furthermore, upon ofloxacin exposure the dim cells proved to be tolerant, confirming that they were, indeed composed by persister cells (**Figure 8**). Although effective, this method has its limitations, mainly those associated with the dilution of the population in the buffer (*e.g.*, both cell and medium concentration changes; population regrowth, which results in a decrease in persister levels) (Lewis, 2010). Nevertheless, a transcriptome of the sorted persisters was obtained in this study (Shah et al., 2006).

Regardless of the persister isolation method applied, the resultant transcriptome reveals a downregulation in the transcription of genes involved in energy production and non-essential functions such as flagellar synthesis (Shah et al., 2006). These results were thought to indicate that persisters are in a truly 'inactive' state, consistent with their phenotype of non- or slow-growth (Keren et al., 2004b; Shah et al., 2006). Additionally, specific overexpressed genes were identified that might

contribute, as well, to the 'inactive' persister phenotype: Rmf, a stationary state inhibitor of translation; SulA, an inhibitor of septation; and toxin–antitoxin (TA) loci RelBE, DinJ and MazEF (Keren et al., 2004b; Shah et al., 2006). The last ones are found on plasmids, where they constitute a maintenance mechanism. Typically, the toxin is a stable protein that inhibits an important cellular function (e.g., translation; replication) and forms an inactive complex with the degradable antitoxin – TA module. After segregation, if a daughter cell does not receive a plasmid the antitoxin level decreases due to proteolysis; in this situation, the toxin either kills the cell or inhibits propagation (Lewis, 2010).
Consider, as an example, *MazF* and *RelE*. These two unrelated toxins, found in *E. coli*, induce stasis by promoting mRNA cleavage, which will, ultimately, inhibit translation. Furthermore, this condition can be reversed by expression of *MazF* and *RelE* respective antitoxins (Lewis, 2010). These TA modules particularities make them promising candidates for persister genes.

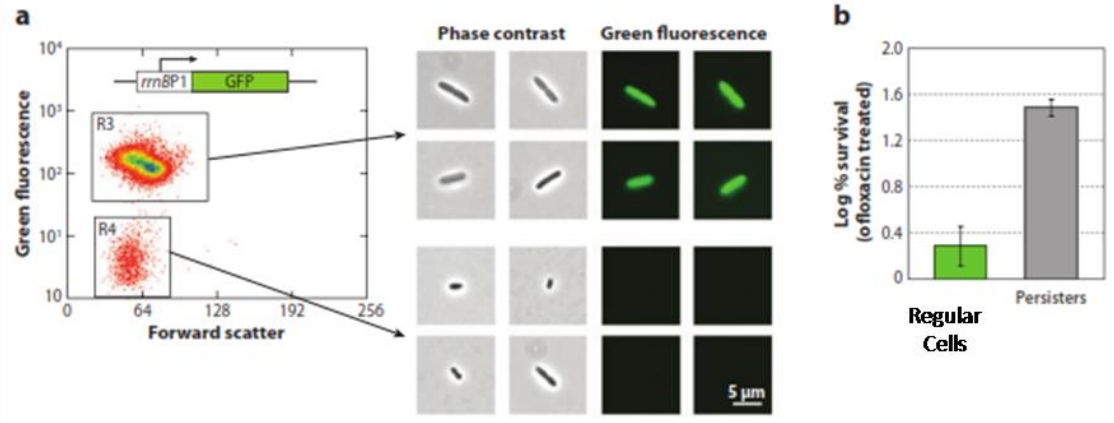

Figure 8 – Persister-cells isolation using degradable GFP expression. **a)** An *Escherichia coli* strain expressing a degradable GFP under the control of a ribosomal promoter was cultured to exponential state and the cells were sorted based on the green signal. The large majority of green cells is composed by regular cells, whereas the small subpopulation is composed by dim cells that apparently have diminished translation – persister cells. **b)** Exposure of these two subpopulations to antibiotics confirmed the nature of the dim fraction of cells, composed by tolerant persisters (grey bar) rather than regular cells (green bar). Adapted from (Shah et al., 2006).

2.5. Methods to Induce, Grow and Assess Persister Formation *in vitro*

In one of the first *in vitro* studies, in 1983, Moyed and Bertrand used distinct antibiotics (ampicillin, phosphomycin, and cycloserine) to induce the formation of persister cells in *E. coli* K12 MH21. For that, *E. coli* K12 MH21 cells were suspended in both rich and minimal medium (Moyed and Bertrand, 1983). The rich medium contained tryptone (10 $mg.mL^{-1}$), yeast extract (5 $mg.mL^{-1}$) and NaCl (8

mg.mL^{-1}); when necessary the medium was supplemented with diaminopimelic acid (DAP) (30 µg.mL^{-1}). The minimal medium used was M9; and in this case required vitamins (1 µg.mL^{-1}), amino acids (40 µg.mL^{-1}), and purines (20 µg.mL^{-1}) were added. For both mediums used, the growth of *E. coli* K12 MH21 cells was monitored by optical density at 540 nm and, at the given times, log-phase cultures were collected. These cultures were successively, and differently, challenged (100 µg.mL^{-1} ampicillin; 50 µg.mL^{-1} phosphomycin; and 50 µg.mL^{-1} cycloserine) until complete cell lysis was obtained (*ca.* 1.5-2 h). First, each of the mutants was exposed to a given antibiotic and concentration. This cycle was then repeated after the survivors had resumed exponential growth. The third and last cycle of antibiotic exposure occurred by plating the remaining survival cells in plaques containing the respective antibiotic in the same concentration used for the previous cycles. After 20 h, each plate was sprayed with a solution of penicillinase (*ca.* 2,000 to 3,000 U.mL^{-1}). Colonies appeared after another 20 h of incubation. These successive antibiotic exposure cycles allowed Moyed and Bertrand to produce and isolate *E. coli* K12 MH21 persister cells (Moyed and Bertrand, 1983).

Since then, many approaches and hypotheses have been tested for the *in vitro* induction of persisters including: relatively high temperature (42^{o}C) for long periods (20h) (Scherrer and Moyed, 1988), biocides (Spoering and Lewis, 2001), acidic stress (pH=3.0) (Booth, 2002), starvation conditions and oxidative stress (Vega et al., 2012), and even genetic approaches combined with antibiotics (Shah et al., 2006). A summary of the different approaches used to induce persister formation *in vitro*, and the respective strains and conditions applied, can be found in **Table 1**.

Regardless of the method used to induce persister formation, it is afterwards necessary to apply a method and/or liquid medium(s) adequate for the growth of the formed persisters. These methods may include microfluidics devices with time-lapse microscopy, namely polydimethylsiloxane (PDMS) flow cells, which can be made using standard photolithography and soft lithography techniques (Golchin et al., 2012); incubation with aeration at 37°C in baffled culture flasks containing an adequate medium (Shah et al., 2006); and 96 polystyrene peg platforms, where dilutions plating can be performed (Spoering and Lewis, 2001).The liquid media used varied between tryptic soy broth (TSB) (Singh et al., 2009), Mueller-Hinton broth (MHB) (Kostenko et al., 2007), Luria-Bertani (LB) broth (Keren et al., 2004a) and many others. A summary of different approaches, stating both methods and liquid media composition, adequate for the growth of *in vitro* induced persisters, and the respective strains and conditions applied, can be found in **Table 2**.

Methods to assess persistence *in vitro* have also been evolving over the past few years. From the simplest spread-plate technique (Johnson and Levin, 2013; Keren et al., 2004a; Kostenko et al., 2007; Singh et al., 2009; Udekwu and Levin, 2012), to the more complexes flow cytometry and fluorescence activated cell sorting (FACS) (Golchin et al., 2012; Shah et al., 2006; Vega et al., 2012), and even the far more laborious genome-wide expression profiling method (Shah et al., 2006; Vega et al., 2012) (**Table 3**).

Table 1 – Summary of the different approaches used to induce persister formation *in vitro*, and the respective strains and conditions applied.

Strain	Compound / Method	[Compound] (μg/mL)/Value	Persisters (%)	t* (h)	Reference
S. aureus	Ciprofloxacin	5 (10*MIC)	0.01	8	*(Johnson and Levin, 2013)*
	Gentamicin	15 (10*MIC)	0.001		
	Oxacillin	1 (5*MIC)	1		
	Vancomycin	5 (2.5*MIC)	0.1		
***S. aureus* PS80**	Linezolid	20*MIC	10	24	(Udekwu and Levin, 2012)
	Ciprofloxacin		0.01		
	Gentamycin	50*MIC	0.001		
	Daptomycin		≈ 100		
	Oxacillin	100*MIC	0.1		
	Vancomycin		10		
***S. aureus* ATCC 29213**	Oxacillin	100	1	48	*(Singh et al., 2009)*
	Cefotaxime		0.1		
	Amikacin		0.001		
	Ciprofloxacin		0.001		
	Vancomycin		1		
***S. aureus* UOC18**	Vancomycin	1024	0.01	24	*(Kostenko et al., 2007)*
M. smegmatis	Rifampicin	350 (10*MIC)	Not shown	4	*(Golchin et al., 2012)*
***P. aeruginosa* PAO1**	Carbenicillin	600 (20*MIC)	≈ 100	6	*(Spoering and Lewis, 2001)*
	Ofloxacin	15 (30*MIC)	0.1		
	Tobramycin	1000 (1000*MIC)	1		
	Biocide (Peracetic Acid)	Biphasic killing kinetic was not observed			

E. coli	Ofloxacin	5	0.001	3	*(Vega et al., 2012)*
	Ampicillin	100	0.01		
	Kanamycin	10	1		
	Starvation (Indole) + Ofloxacin	5	0.01	3	
	Starvation (Indole) + Ampicillin	100	1		
	Starvation (Indole) + Kanamycin	10	50		
	Oxidative Stress (H_2O_2) + Ofloxacin	5	1	1	
E. coli **MG1655-ASV**	Ofloxacin	5	25	3	*(Shah et al., 2006)*
	ygiU Overexpression + Ofloxacin	5	80	3	
	ygiU Overexpression + Mitomycin C	10	0.001		
	ygiU Overexpression + Tobramycin	20	0.0001		
	ygiU Overexpression + Cefotaxime	100	65		
E. coli **K12**	Ampicillin	100	0.01	3	*(Keren et al., 2004a)*
	Ofloxacin	5			
E. coli **CRA6353**	Acid Stress	pH 3 (in McIlvaine's citrate)	0.01	1	*(Booth, 2002)*
E. coli **K12**	Ampicillin	100	17	20	*(Scherrer and Moyed, 1988)*
	Temperature	42°C	30		
E. coli **W7**	CGP 17520	0.8	8	6	*(Tuomanen et al., 1986)*
E. coli **K12 HM21**	Ampicillin	100	25	4	*(Moyed and Bertrand, 1983)*
	Phosphomycin	50	25		
	Cycloserine	50	50		

* Time needed to obtain persisters (*i.e.* time in contact with the 'inducer agent').

Table 2 – Summary of the different methods and liquid media composition, adequate for the growth of *in vitro* induced persisters, and the respective strains and conditions applied.

Strain	Method / Liquid Medium [#]	CFU / mL (method) [+]	Reference
***S. aureus* ATCC 29213**	TSB) / TSA plaques	10^9 (drop plating)	*(Singh et al., 2009)*
***S. aureus* ATCC 29213** ***S. aureus* UOC18**	MHB	10^7 (colony counting)	*(Kostenko et al., 2007)*
M. smegmatis	Microfluidics Device: PDMS flow cell	10^7 (OD_{600nm})	*(Golchin et al., 2012)*
***E. coli* MG1655-ASV**	Incubation in Baffled Culture Flasks with LB	10^7 (colony counting)	*(Shah et al., 2006)*
E. coli	Microfluidics Chemostat using LB or M9 Minimal Medium (supplemented with Casamino Acids and Glucose)	Not shown	*(Vega et al., 2012)*
***E. coli* K12**	LB broth and LB Plates	10^7 (colony counting)	*(Keren et al., 2004a)*
***P. aeruginosa* PAO1**	96 Polystyrene Pegs Platform (dilutions plating)	10^8 (colony counting)	*(Spoering and Lewis, 2001)*

[#] Method / liquid medium used to grow persisters.

[+] CFU obtained by colony counting or by optical density measurements at a wavelength of 600 nanometers.

Table 3 – Summary of different methods to assess persistence *in vitro*, and the respective strains and conditions applied.

Strain	Method	Method Description	Reference
S. aureus	Spread-Plate Technique		*(Johnson and Levin, 2013)*
S. aureus PS80	Spread-Plate Technique		*(Udekwu and Levin, 2012)*
***S. aureus* ATCC 29213**	Spread-Plate Technique		*(Singh et al., 2009)*
***S. aureus* UOC18**	Spread-Plate Technique		*(Kostenko et al., 2007)*
M. smegmatis	GFP Expression/ Non-expression	Confocal Laser Microscopy	(Golchin et al., 2012)
E. coli	Flow Cytometry and FACS Genome-wide Expression Profiling	Fluorescence-based Sorting (GFP) Affymetrix arrays	*(Vega et al., 2012)*
***E. coli* MG1655-ASV**	Genome-wide Expression Profiling FACS + Epifluorescent Microscopy	Spotted Microarrays MoFlo (DakoCytomation) Cell Sorter	(Shah et al., 2006)
***E. coli* K12**	Spread-Plate Technique		*(Keren et al., 2004a)*

3. MATERIALS AND METHODS

3.1. Bacterial Strains and Growth Conditions

Strain *Staphylococcus aureus* ATCC 25923 was kindly provided by the Unit of Mycobacteriology of the Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa. Strain *Rhodococcus erythropolis* DCL14 was isolated by the Division of Industrial Microbiology of the Wageningen University, The Netherlands (van der Werf et al., 1999). It is deposited and maintained at the Institute for Biotechnology and Bioengineering, Lisbon, Portugal. Both *S. aureus* and *R. erythropolis* cells were suspended in 20 mL of Mueller Hinton Broth (MHB; Fluka) in 100mL Erlenmeyer flasks. *S. aureus* cells were cultured at 37°C and 200 rpm whilst *R. erythropolis* grew at 28ºC and 200 rpm on incubators Agitorb 200 (Aralab, Portugal). The growth of the planktonic cells was monitored by optical density measured using a Merck-Hitashi spectrophotometer at 600 nm.

3.2. Reagents and Antibiotics

Mueller Hinton powder was purchased from Sigma-Aldrich (Fluka Analytical). MHB was prepared by diluting 23 g of power in 1 L of distilled water. After autoclaving at 121ºC, the broth was stored at 4°C for no more than 1 week. The agar plates were prepared by adding the following components per liter of demineralized water in the order described next: 4 mL of 100x concentrated Mineral Medium; 4 mL of 100x concentrated Phosphate Buffer; 6 $g.L^{-1}$ of agar; 2 $g.L^{-1}$ of glucose; 1.4 $g.L^{-1}$ of yeast extract (Difco). The 100x concentrated Mineral Medium solution was obtained by adding, per liter of demiwater, the salts in the order described next: 1 g EDTA; 0.2 g $ZnSO_4.7H_2O$; 0.1 g $CaCl_2.2H_2O$; 0.5 $FeSO_4.7H_2O$; 0.2 g $Na_2MoO_4.2H_2O$; 0.2 g $CuSO_4.5H_2O$; 0.4 g $CoCl.6H_2O$; 0.1 g $MnCl_2.4H_2O$; 200 g $(NH_4)_2SO_4$; 10 g $MgCl_2.6H_2O$ (salts purchased from Sigma-Aldrich). 100x concentrated buffer solution (50 mM, pH = 7.0) was prepared by adding, per liter of demiwater, the components in the following order: 155 g K_2HPO_4; 85 g $NaH_2PO_4.H_2O$ (salts purchased from Sigma-Aldrich). All media used were sterilized in an autoclave at 121°C for 15 minutes. Vancomycin (Vancomycin hydrochloride from *Streptomyces orientalis* with a potency ≥ 900 $\mu g.mg^{-1}$) and teicoplanin (from *Actinoplanes teichomyceticus* with a purity ≥ 80%) were purchased from Sigma-Aldrich. Both antibiotics were used at concentrations of 50 and 100 $\mu g.mL^{-1}$ in milli-Q water.

3.3. Determination of the Minimal Inhibitory Concentration (MIC)

The MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2014). In summary, antibiotics were serially diluted in two-fold steps (from 100 $\mu g.mL^{-1}$ to 0.037 $\mu g.mL^{-1}$) in 96-well microplates (Sarstedt Inc., Newton, USA) in Mueller-Hinton medium. To 150 µL of medium containing the antibiotic in each well, 50 µL of an exponentially growing cell culture diluted to 0.5 McFarland standard was added. The microplates containing *S. aureus* cells were incubated at 37ºC whilst *R. erythropolis* cells were incubated at 28ºC. The MIC was determined for each antibiotic by measuring the optical density of cell cultures after 16 and 24 h of exposure on a SpectraMax® Plus 384 Microplate Reader spectrophotometer from Molecular Devices (Silicon Valley, CA, USA) at 600 nm. At least two independent tests were performed. The MICs determined for each bacterium and antibiotic were the following: $MIC_{vancomycin\ S.\ aureus}$ = 0.59 $\mu g.mL^{-1}$ and $MIC_{teicoplanin\ S.\ aureus}$ = 0.39 $\mu g.mL^{-1}$; $MIC_{vancomycin\ R.\ erythropolis}$ = 1.17 $\mu g.mL^{-1}$ and $MIC_{teicoplanin\ R.\ erythropolis}$ = 1.56 $\mu g.mL^{-1}$.

3.4. Time-Dependent Killing

To 1 mL of exponentially growing cells, a given concentration of antibiotic was added in 12 mL pyrex tubes. The tubes containing *S. aureus* were incubated on a shaker at 200 rpm and 37°C for a total of 6 h whilst those containing *R. erythropolis* were incubated at 28ºC and 200 rpm. At certain time intervals (30 min, 1, 2, 3, 4, 5 and 6 h), a 20 µL sample was collected and spread on an agar plate for determination of CFU. The CFU counts were performed after 16-18 h according to CLSI guidelines (CLSI, 2014) or after 37-38 h of incubation when growth was slower.

3.5. Growth after Antibiotic Exposure

After challenging each culture with 50 and 100 $\mu g.mL^{-1}$ vancomycin or teicoplanin for 3, 4, 5 or 6 h (as stated in **3.4. Time-Dependent Killing**) 1 mL of culture was collected and added to 19 mL of fresh MHB in 100 mL Erlenmeyers. Growth was promoted for each strain at the conditions previously stated and monitored by measuring the optical density of the media at 600 nm until stationary phase was reached.

3.6. Cell Viability Analysis

Cell viability was assessed, upon exposure to 50 and 100 $\mu g.mL^{-1}$ vancomycin or teicoplanin, by fluorescence microscopy using an Olympus CX40 microscope equipped with an Olympus U-RFL-T burner and an U-MWB mirror cube unit (excitation filter: BP450-480; barrier filter: BA515). Cells were stained using a LIVE/DEAD® *Bac*Light™ Bacterial Viabillity Kit (Molecular Probes; Life Technologies;

Thermo Fisher Scientific) and images were captured by an Evolution™MP5.1 CCD colour camera using the software Image-Pro Plus (both from Media Cybernetics, Inc., USA). Image analysis was performed as described previously (de Carvalho et al., 2003). At least 15 images were taken from each sample.

3.7. Enzymatic Activity Analysis

Semiquantification of the enzymatic activities of *S. aureus* cells before and after exposure to antibiotics, and subsequent growth in fresh medium after drug removal, was assessed using a API ZYM® kit from BioMérieux (France). According to the manufacturer, a semiquantitative scale was used to classify color intensity: 0 (no activity) to 6 (strong activity).

3.8. Oxygen Consumption Analysis

To determine the metabolic activity of the cells, oxygen consumption analyses were performed on Oxodish® OD24 microtiter plates, using a SDR Sensor Dish® Reader (all from PreSens, Germany). Each bacterium was exposed to the adequate antibiotic concentration and compared to non-exposed cells. To test the effect of low antibiotic concentrations, *S. aureus* cells were exposed to both vancomycin and teicoplanin at concentrations ranging between 0.0020 and 1 $\mu g.mL^{-1}$, whilst for high antibiotic concentrations 50 and 100 $\mu g.mL^{-1}$ were used. Growth of the cells on fresh medium was assessed after 3, 4, 5 and 6 h of pre-exposure to each antibiotic and concentration. The assays were monitored for up to 48 h. The plates containing *S. aureus* cells were incubated at 37ºC and 200 rpm whilst *R. erythropolis* cells were incubated at 28ºC for 200 rpm. Data acquisition was performed by the software SDR_v37 from PreSens.

3.9. Lipids Analysis

The FAMEs analysis was performed by gas chromatography (GC). For that, 1 mL of cell suspension was collected from each culture and the cells were recovered by centrifugation at 10,000 g for 5 minutes in 1.5 mL eppendorfs (Eppendorf). Cells were then washed by vortex with 1 mL of milli-Q water and recovered by performing a second centrifugation step at 10,000 g for 5 minutes. The fatty acids contained in the cell pellet were simultaneously extracted and methylated using the Instant FAME™ procedure from MIDI, Inc. (Newark, USA). The FAMEs were analysed on a 6890N gas chromatograph from Agilent Technologies (Palo Alto, CA, USA), with a FID and a 7683 B series injector, and equipped with a 25 m long Agilent J&W Ultra 2 capillary column from Agilent. The gas chromatograph was programmed and controlled by the MIDI Sherlock software package, version 6.2. The FAMEs were identified by the MIDI software, using MIDI calibration standards, a methyl cis-11-

octadecenoate standard solution from Sigma-Aldrich and confirmed by using two qualitative standards, one containing a mixture of bacterial FAMEs and another of polyunsaturated fatty acids (PUFA), both from Supelco. The average error associated with the GC quantification of each FAMEs composition was ± 2.2 %, quoted for a confidence interval of 99.5 %. Errors were calculated based on seven independently prepared standard solutions.

3.10. Zeta Potential Analysis

The Zeta Potential analysis was performed using a Zetasizer Nano ZS from Malvern Instruments Ltd. (Malvern, UK). For that, 20, 40 or 60 µL of cell suspension was collected from each culture and added to 2 mL of a 1.012 $\mu g.mL^{-1}$ potassium nitrate (KNO_3) solution in 2 mL eppendorfs. Data acquisition was carried out by the software Zetasizer 7.10 from Malvern Instruments Ltd. (Malvern, UK). The assays were done in triplicate and the results represented are average values.

3.11. FTIR Analysis

To determine the effect of antibiotic exposure to the cells, a Fourier transform infrared (FTIR) spectroscopy analysis was performed on both *S. aureus* and *R. erythropolis* cells before and after a 4 h exposure to antibiotics and subsequent growth in fresh medium after drug removal. Cells were collected by centrifugation at 4,000 g for 10 min and the pellets suspended in demineralized water to obtain an optical density of *ca.* 64. Samples containing 20 µL from each prepared cell suspension were plated on a 96-well zinc selenium plate from Bruker Optics® (Billerica, Massachusetts, USA) and dehydrated in a vacuum desiccator containing silica until full dehydration was achieved, confirmed by visual inspection (*ca.* 45 min). The readings were performed by transmission mode using a HTS-XT module coupled to a Vertex-70 spectrometer from Bruker Optics® (Billerica, Massachusetts, USA), with a resolution of 4 cm^{-1}. A total of 40 scans were performed per sample. The data were analyzed after a spectral pre-processing by a Multiplicative Scatter Correction (MSC), previously implemented (Geladi et al., 1985; Isaksson and Næs, 1988; Naes et al., 1990), and applied using Chemometric data preprocessing software from MATLAB® Central (USA) (http://www.mathworks.com/matlabcentral/fileexchange/30765-chemometric-data-preprocessing). The pre-processing was followed by a smoothing using the Savitzky-Golay algorithm ((Savitzky, 1989; Savitzky and Golay, 1964)) and a 2nd order derivative via the same algorithm. The final multivariate analysis was performed using Partial Least Squares (PLS) regression or Discriminant Analysis software from MATLAB® Central (USA) (http://www.mathworks.com/matlabcentral/fileexchange/30685-pls-regression-or-discriminant-analysis--with-leave-one-out-cross-validation-and-prediction/content/PL S/pls.m).

4. RESULTS AND DISCUSSION

To promote the formation of persister cells, the cultures containing each bacterium were exposed to high concentrations of each of the antibiotic tested. During exposure to the antibiotic a time-dependent killing curve was expected with only a partial number of cells surviving. If the survival population comprised persister cells, the growth curve obtained after placing these cells in fresh medium should be similar to the initial non-stressed population. The experiments were thus conducted as follows:

- *S. aureus* and *R. erythropolis* cells were grown in MHB until growth reached mid-exponential phase;
- vancomycin/teicoplanin was added at the adequate concentration and its effect on the bacterial population was assessed during a total of 6 hours;
- the surviving cells were recovered and placed in fresh MHB containing no drug and their growth was followed during for 24 hours.

4.1. Bacterial Cultures under Non-Stress Conditions

4.1.1. Normal Growth

In Mueller-Hinton broth, both *S. aureus* and *R. erythropolis* reached the exponential phase in less than 3 h of culture. Duplication times (t_d) of approximately 0.71 and 0.85 h^{-1} were obtained for *S. aureus* and *R. erythropolis*, respectively (**Figure 9**).

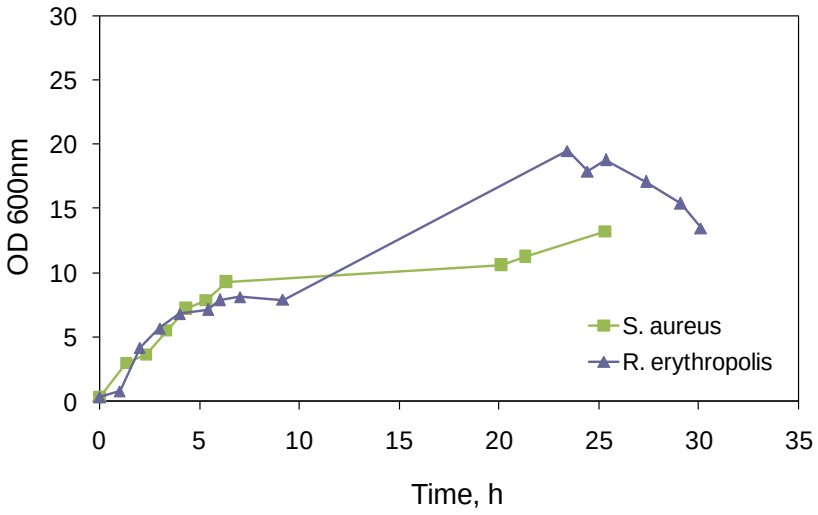

Figure 9 – Growth curves of *S. aureus* (green line) and *R. erythropolis* (purple line) in MHB.

4.1.2. Lipids Analysis

It is now known that bacterial cells alter their fatty acids (FA) profile once they reach the stationary phase and in response to environmental changes (Bezbaruah et al., 1988; de Carvalho et al., 2005; Murínová and Dercová, 2013). In the present work, the lipid composition of the cells was studied in several growth stages (**Figure 10** and **Figure 11**).

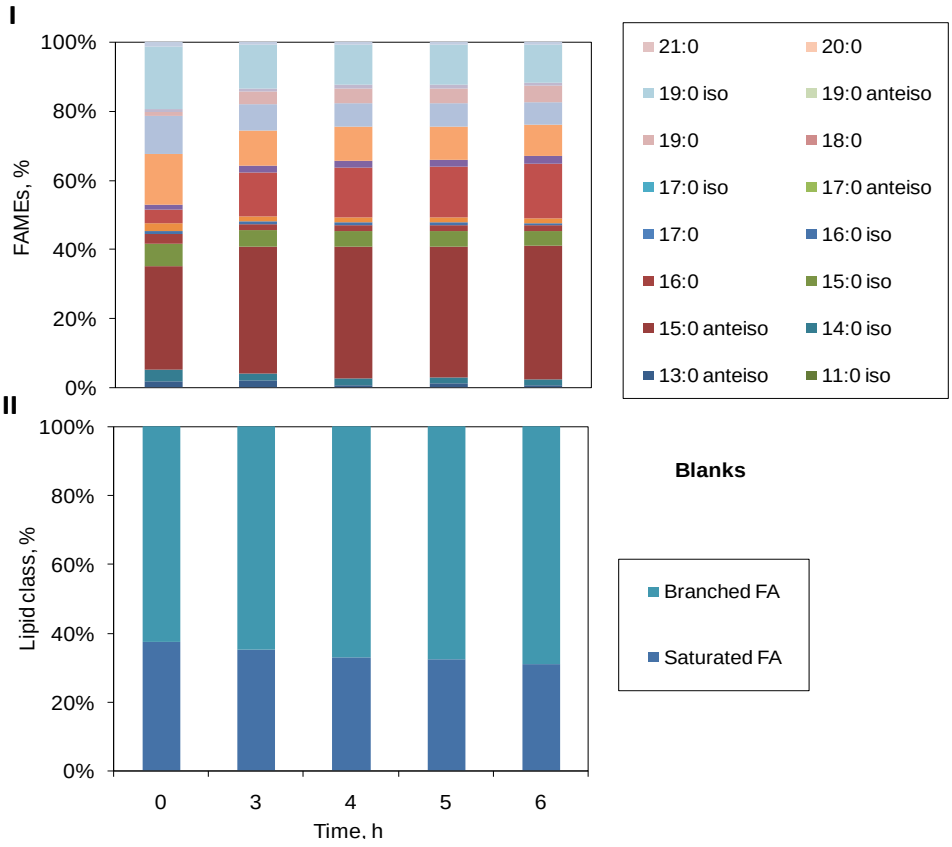

Figure 10 (I) and (II) – Lipid composition of *S. aureus* during cell growth. **(I)** Composition in fatty acids (only those present at concentrations higher than 1% are shown) and **(II)** according to the type of fatty acid molecule.

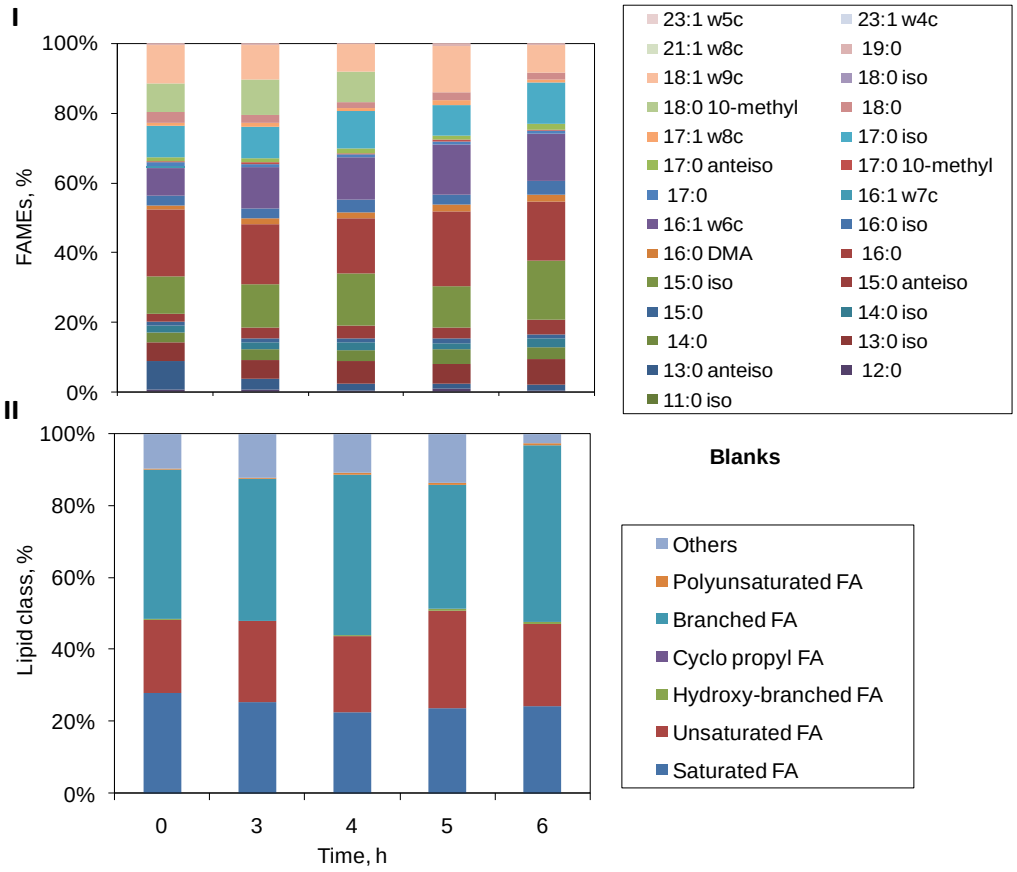

Figure 11 (I) and (II) – Lipid composition of *R. erythropolis* during cell growth. **(I)** Composition in fatty acids (only those present at concentrations higher than 1% are shown) and **(II)** according to the type of fatty acid molecule.

Both *S. aureus* and *R. erythropolis* cells decreased the percentage of saturated FA while increasing the percentage of branched FA with increasing culture age (**Figure 10 (II)** and **Figure 11 (II)**). Saturated cyclopropyl-branched and hydroxyl-substituted FA were present at concentrations lower than 2% in both stains and unsaturated FA were also at such low percentage in *S. aureus* (**Figure 10** and **Figure 11**). Furthermore, the analysis of the values presented in **Figure 10 (I)**, shows that the more significant decreases were observed at the levels of saturated FA such as 18:0 (decreased from 14.19 to 9%), 19:0 (from 10.64 to 6.45%), and 20:0 (from 17.68 to 10.85%), in the case of *S. aureus* (**Figure 10 (I)**); and of 18:0 10-methyl (decrease from 8.29 to 0%) in the case of *R. erythropolis* (**Figure 11 (I)**). On the other hand, the more significant increases were observed in the levels of branched FA such as 15:0 *anteiso* (increased from 29.03 to 38.26%), 17:0 *anteiso* (from 3.82 to 15.60%) and 19:0 *anteiso* (from 1.18 to 4.61%) in the case of *S. aureus* (**Figure 10 (I)**); and of 13:0 *iso* (from 5.22 to 7.19%), 15:0 *iso* (from 10.68 to 16.26%) and 17:0 *iso* (from 8.92 to 11.58%) in the case of *R. erythropolis* (**Figure 11 (I)**). These results thus confirm what has been reported: bacterial cells tend to decrease the degree of saturation of membrane phospholipids over time under non-stress conditions (Murínová and Dercová, 2013).

4.1.3. Zeta Potential Analysis

It has been reported that bacterial cells adjust the surface net charge in response to environmental changes and to the presence of toxic compounds (de Carvalho, 2012; de Carvalho et al., 2009). In the present study, the zeta potential of the cells decreased with increasing age of the culture for both *S. aureus* and *R. erythropolis* cells: between -14.8 and -18.5 mV for the former (**Figure 12**) and -24.9 and -31.4 mV for the latter (**Figure 13**), after 6 h of culture.

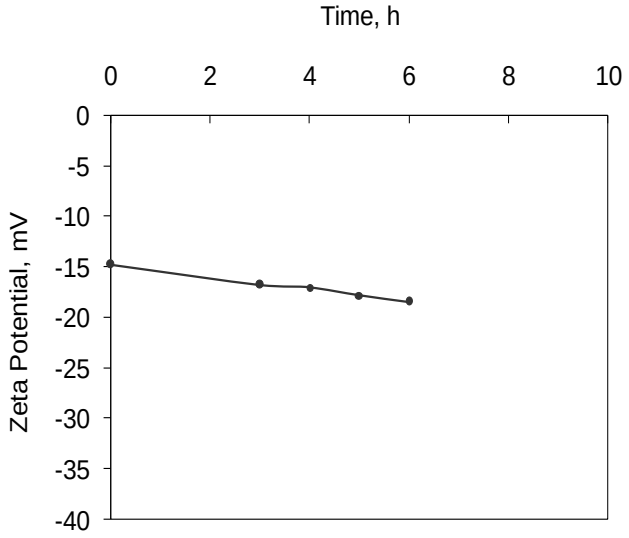

Figure 12 – Effect of the age of the culture of *S. aureus* in the net surface charge of the cells.

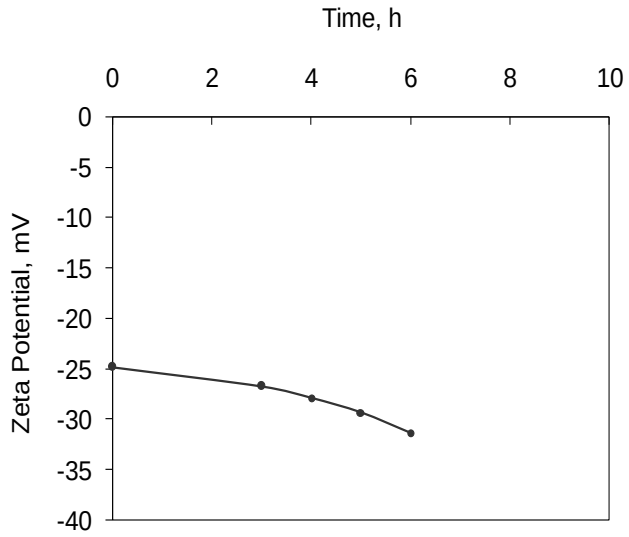

Figure 13 – Effect of the age of the culture of *R. erythropolis* in the net surface charge of the cells.

4.1.4. Oxygen Consumption Analysis

The metabolic activity of bacterial cells varies according to the growth stage of the cells: cells during the exponential phase require much more energy than during the stationary phase, which may be assessed by the oxygen consumption rate.

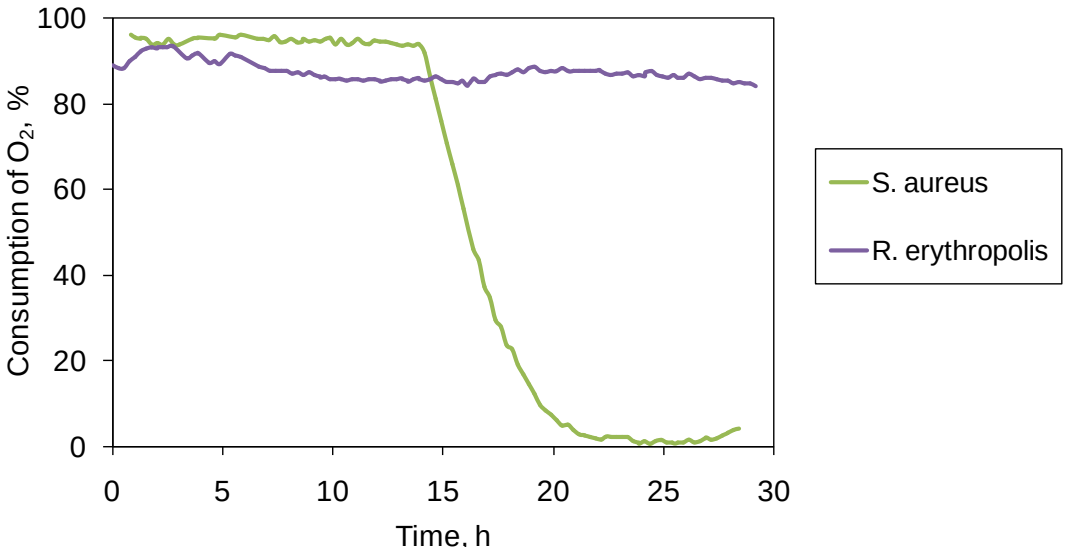

Figure 14 – Percentage of oxygen consumed during *S. aureus* (green line) and *R. erythropolis* (purple line) growth.

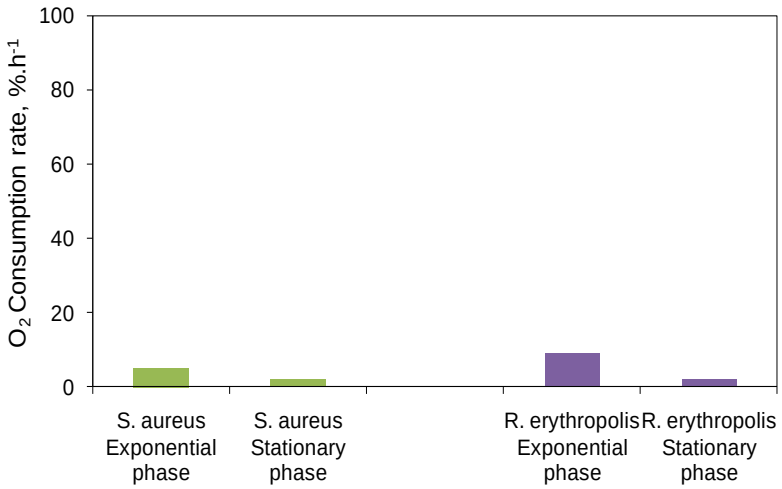

Figure 15 – *S. aureus* (green bars) and *R. erythropolis* (purple bars) oxygen consumption rates, according to the growth phase.

The analysis of the oxygen consumption along time of culture reveals, as expected, an initial phase during which cells consumed very high levels of oxygen. This phase corresponds to the cells in the exponential growth phase, during which they consume high amounts of oxygen. Accordingly, after that period, the levels of consumption of oxygen rapidly decrease in the case of *S. aureus*, until the oxygen in the medium reached values near to 100%. By that time, *S. aureus* cells have likely reached the stationary phase of growth. For *R. erythropolis*, however, that pattern was not observed. Instead, *R. erythropolis* cells maintained high consumption rates during the period analyzed. In fact, *R. erythropolis* cells, and contrary to *S. aureus*, have shown to continue growing, although at lower rates than those observed during the exponential phase, even when apparently the stationary phase was reached (**Figure 9**). The results thus suggest that *R. erythropolis* comprise longer and more energy consuming growth comparatively to that of *S. aureus*, resulting in higher oxygen consumption rates (**Figure 15**).

4.2. Bacterial Cultures under Stress Conditions

As mentioned above, when a population is exposed to stress conditions, *e.g.* to a bactericidal antibiotic present at high concentrations, regular cells die whereas persisters survive. Therefore, taking samples and plating them for CFU over time from a culture exposed to a given antibiotic should produce a biphasic pattern, with a distinct plateau of surviving persisters. For that, and since persister-cells formation/isolation is generally increased upon exposure to high doses (high MIC equivalents) of bactericidal antibiotics (Moyed and Bertrand, 1983), high concentrations must be used.

4.2.1. Determination of the MIC

To select the concentration of each antibiotic that would allow the formation/isolation of high levels of persister cells, and since that process is generally favored upon exposure to high doses (high MIC equivalents) of bactericidal antibiotics (Moyed and Bertrand, 1983), the MICs of each one of the two tested antibiotics were determined for both *S. aureus* and *R. erythropolis*. The optical density data used are presented in **APPENDIX** (**Erro! A origem da referência não foi encontrada.** and **Erro! A origem da referência não foi encontrada.** for *S. aureus* and respectively for vancomycin and teicoplanin; and **Erro! A origem da referência não foi encontrada.** and **Erro! A origem da referência não foi encontrada.** for *R. erythropolis* and respectively for vancomycin and teicoplanin).

MICs of 0.59 and 0.39 µg.mL^{-1} were obtained, respectively, for vancomycin and teicoplanin in the case of *S. aureus* whilst MICs of 1.17 and 1.56 µg.mL^{-1} were obtained, respectively, for vancomycin and teicoplanin in the case of *R. erythropolis*.

4.2.2. Time-Dependent Killing

The susceptibility of exponentially growing *S. aureus* (**Figure 16**) and *R. erythropolis* (**Figure 17**) cultures to vancomycin and teicoplanin was tested. It should be noted that these two potent bactericidal glycopeptides have a similar mechanism of action, interfering with the late stages of cell wall biosynthesis (Bambeke, 2004; Cazzola et al., 2004)). Vancomycin and teicoplanin form a stoechiometric complex with the D-Ala-D-Ala dipeptide via the formation of five hydrogen bonds with the peptidic backbone, preventing both the transglycosylation and transpeptidation reactions and thus the incorporation of the precursors into the rigid bacterial cell wall peptidoglycan (Allen et al., 1996; Bambeke, 2004; Nagarajan, 1991). Therefore, to study the effect of the concentration of these two antibiotics on the Gram-positive bacteria *S. aureus* and *R. erythropolis*, cells were collected and exposed for a total of 6 h to 50 and 100 µg.mL^{-1}. The cell numbers at given times were determined by CFU counts and used to calculate both the percentage of survived cells (**Figure 16** and **Figure 17**) and the respective death rates (**Figure 18** and **Figure 19**).

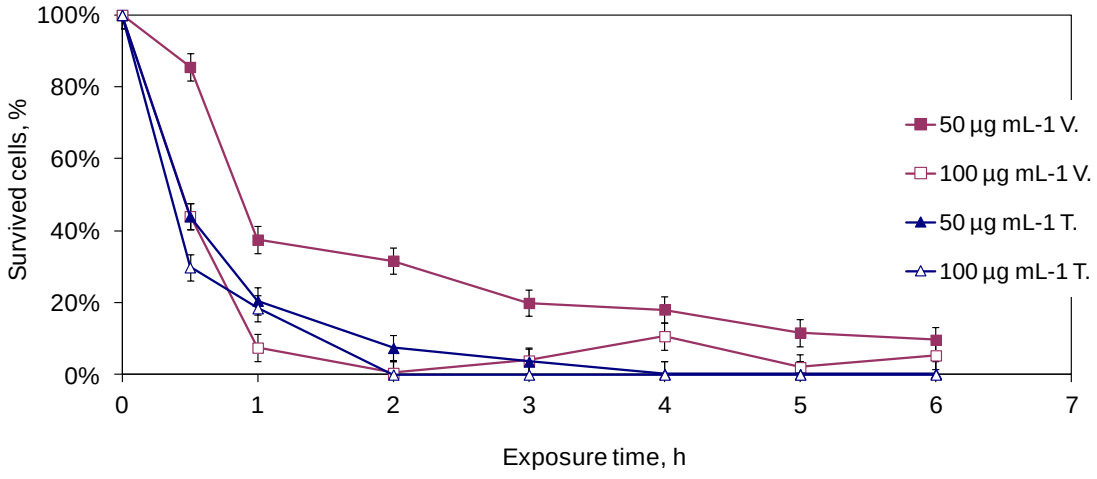

Figure 16 – Percentage of *S. aureus* cells that survived during 6 h of exposure to high concentrations of vancomycin ('V.'; squares) or teicoplanin ('T.'; triangles). Error bars represent the respective standard deviation.

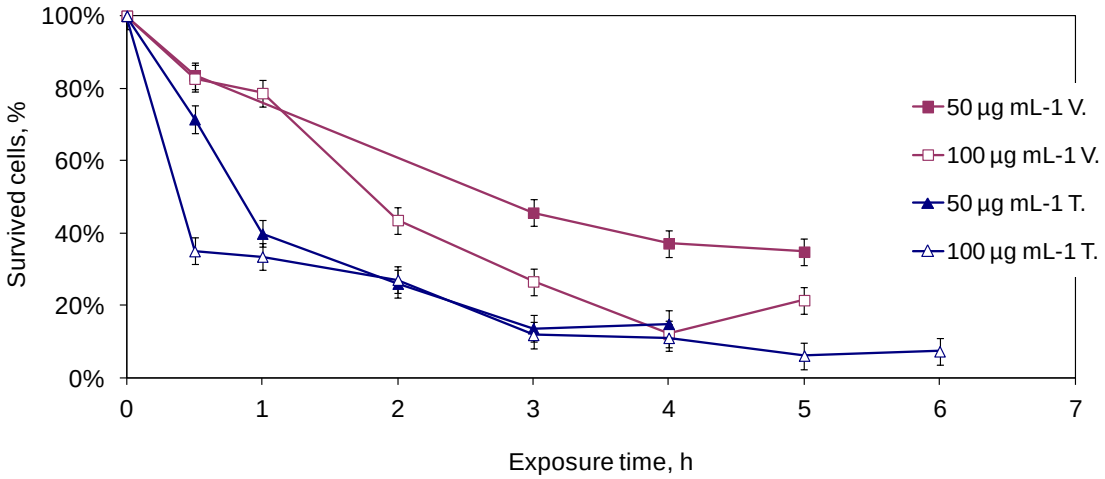

Figure 17 – Percentage of *R. erythropolis* cells that survived during 6 h of exposure to high concentrations of vancomycin ('V.'; squares) or teicoplanin ('T.'; triangles). Error bars represent the respective standard deviation.

The typical biphasic killing patterns (Balaban et al., 2004) were observed for both bacteria and for each tested conditions, showing the survival of the subpopulation of persister cells (**Figure 16** and **Figure 17**). Nevertheless, different survived cells curves, depending on the antibiotic and concentration used were observed, indicating physiological heterogeneity in terms of robustness or fitness within persister subpopulations. This behavior, observed even for antibiotics with identical mechanisms of action such as vancomycin and teicoplanin, suggests that the fraction of cells that survive antibiotic exposure is highly dependent on the specific antibiotic that is used. Similar results led, recently, Johnson and Levin, and later in the same year Hofsteenge *et al.*, to hypothesize that additional physiological changes/mechanisms, rather than only the entrance in the so called 'dormant state', are responsible for different persister phenotypes or levels of induced persistence (Hofsteenge et al., 2013; Johnson and Levin, 2013) (vide section **2.3.4. Persisters – 'Dormant' or Active Cells?**).

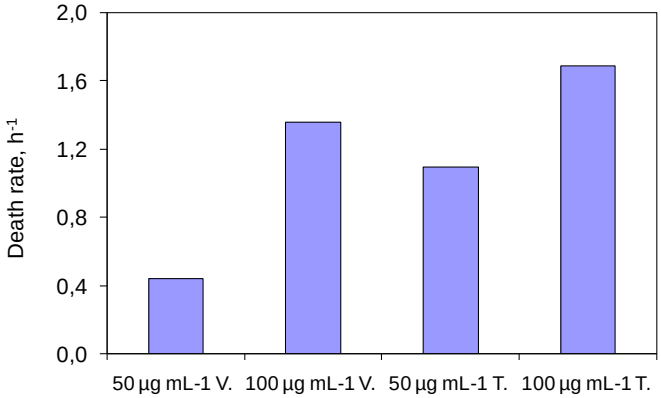

Figure 18 – *S. aureus* death rates determined during 6 h of exposure to high concentrations of vancomycin ('V.') or teicoplanin ('T.').

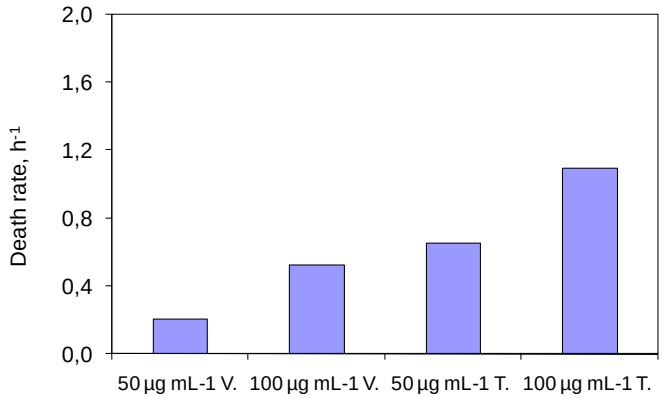

Figure 19 – *R. erythropolis* death rates determined during 6 h of exposure to high concentrations of vancomycin ('V.') or teicoplanin ('T.').

As expected, death rates increased with the antibiotic concentration (**Figure 18** and **Figure 19**). Both the percentage of survived cells and death rates show a higher tolerance, regardless of the tested antibiotic/concentration, for *R. erythropolis* when compared to that of *S. aureus*. This enhanced antibiotic tolerance observed in *R. erythropolis* might suggest the presence of a higher enrichment in persister cells, which might be, in turn, somehow related to its reduced duplication time (t_d = 0.85 h^{-1}) compared to that of *S. aureus* (t_d = 0.71 h^{-1}). According to Hofsteenge *et al.* (Hofsteenge et al., 2013), the different levels of antibiotic tolerance could result from distinct levels of induced persistence, which might vary from antibiotic to antibiotic. It should be noted that teicoplanin already proved to be partially more active than vancomycin against *S. aureus* and enterococci, suggesting an higher persistence induction capacity (Cazzola et al., 2004).

4.2.3. Cell Viability Analysis

To further test the susceptibility of exponentially growing *S. aureus* cultures to vancomycin and teicoplanin, the cell viability was determined during exposure to 50 and 100 µg.mL^{-1} vancomycin or teicoplanin. For that, the number of viable cells (*i.e.*, cells with an intact membrane) was counted and used to calculate the respective percentage of cell viability (**Figure 20** and **Figure 21**).

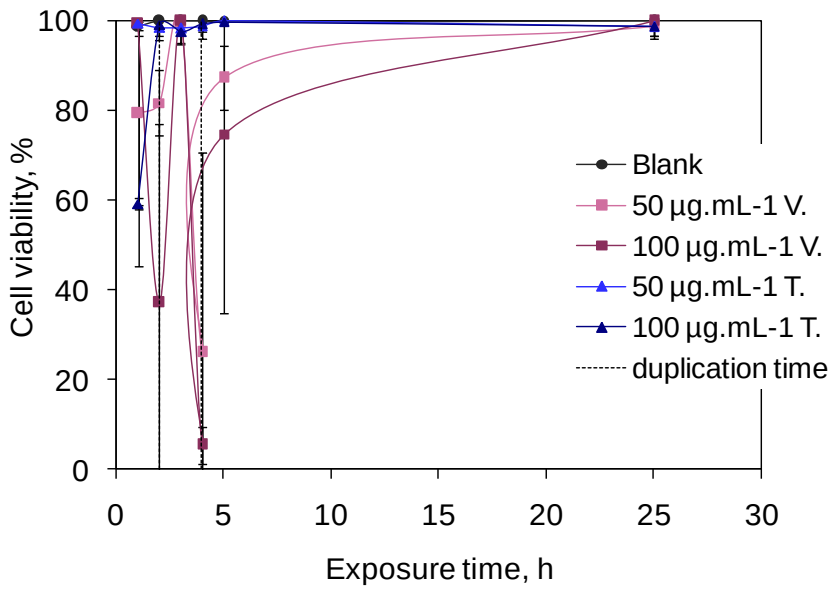

Figure 20 – Percentage of cell viability of *S. aureus* cells during 6 h of exposure to high concentrations of vancomycin ('V.'; squares) or teicoplanin ('T.'; triangles). Error bars represent the respective standard deviation. The 'Blank' curve (circles) represents the non-exposed culture.

The percentage of cell viability (**Figure 20**), calculated from the respective number of viable cells in the overall *S. aureus* population, decreased with time, especially when the duplication time was reached (1.98 and 3.96 h). Nevertheless, the values obtained for the cell viability assay (**Figure 20**) were higher than those obtained for the percentage of survived cells (CFUs) (**Figure 16**). Consider as an example the values obtained at 5 h: 99.65 %, 87.31 %, 100 % and 99.78 %, respectively for 50 µg.mL^{-1} and 100 µg.mL^{-1} vancomycin and 50 µg.mL^{-1} and 100 µg.mL^{-1} teicoplanin, obtained by the cell viability assay (**Figure 20**); and 11.69 %, 2.05 %, 0.26 % and 0 % obtained by CFUs under the same conditions (**Figure 16**). These results can be easily explained by the fact that the bacterial viability kit used in the microscopy assay distinguishes viable (*i.e.*, cells with an intact membrane) from non-viable cells (*i.e.*, cells with a damaged membrane), whilst the CFU counts indicate the number of cells able to divide. Accordingly, the percentage of viable cells obtained by microscopy must always be high than that obtained by CFU counts, since after exposure a given cell might be able to maintain/restore its membrane integrity but not its reproductive capacity.

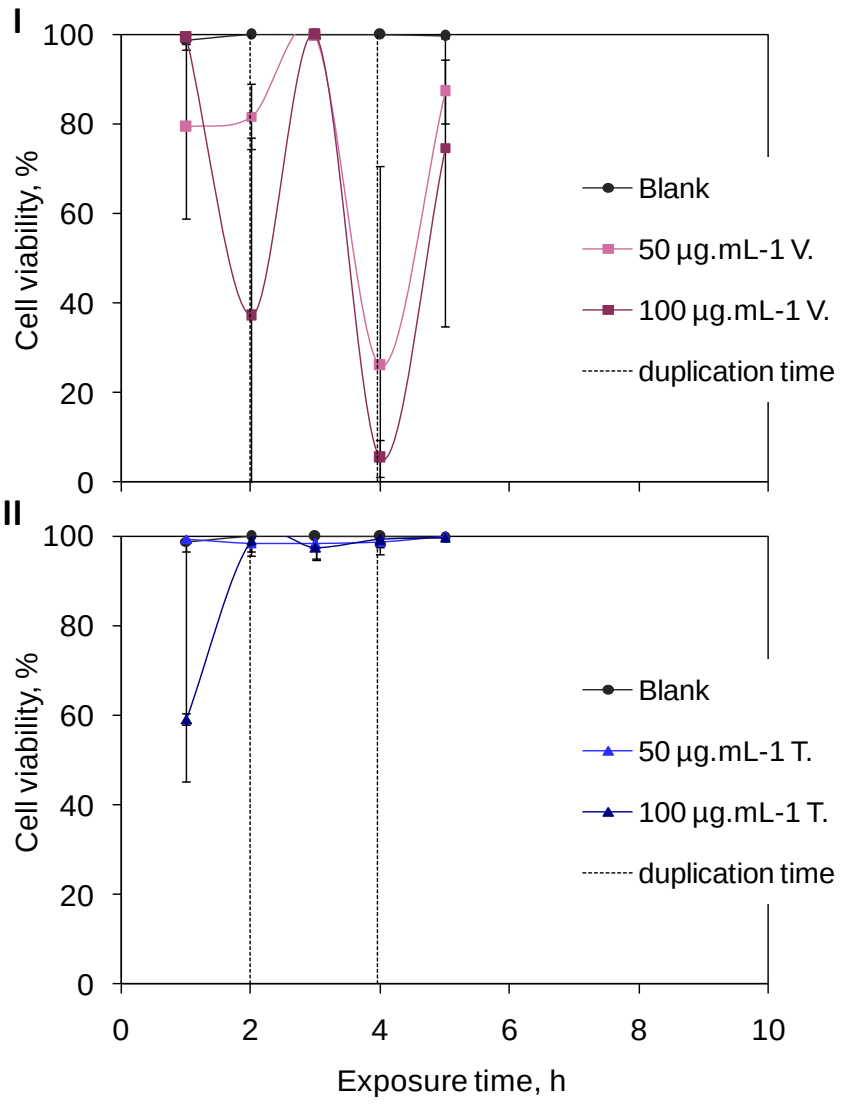

Figure 21 (I) and **(II) –** Percentage of cell viability of *S. aureus* cells during 6 h of exposure to high concentrations of vancomycin ('V.', squares; **I**) or teicoplanin ('T.', triangles; **II**). Error bars represent the respective standard deviation. The 'Blank' curve (circles) represents the non-exposed culture.

4.2.4. FTIR Analysis

Fourier transform infrared (FTIR) spectroscopy has been used, among other areas of research, in the study of bacterial mechanisms of response to environmental stress conditions. In 2011, Alvarez-Ordóñez *et al.* (Alvarez-Ordóñez et al., 2011), reviewed the published experiments, data-processing algorithms and approaches used in this technique to assess bacterial mechanisms of adaptation. The author found evidences of the applicability of this spectroscopy technique in distinct areas, such as the search for mechanisms of bacterial inactivation by food processing technologies and antimicrobial compounds, the monitoring of the spore and membrane properties of foodborne pathogens in

changing environments, the detection of stress-injured microorganisms in food-related environments, the assessment of dynamic changes in bacterial populations, and the study of bacterial tolerance responses. Here, and given the vast range of applications of the technique, the distribution of either exposed or non-exposed *S. aureus* (**Figure 22**) and *R. erythropolis* (**Figure 23**) cells were compared using FTIR spectroscopy.

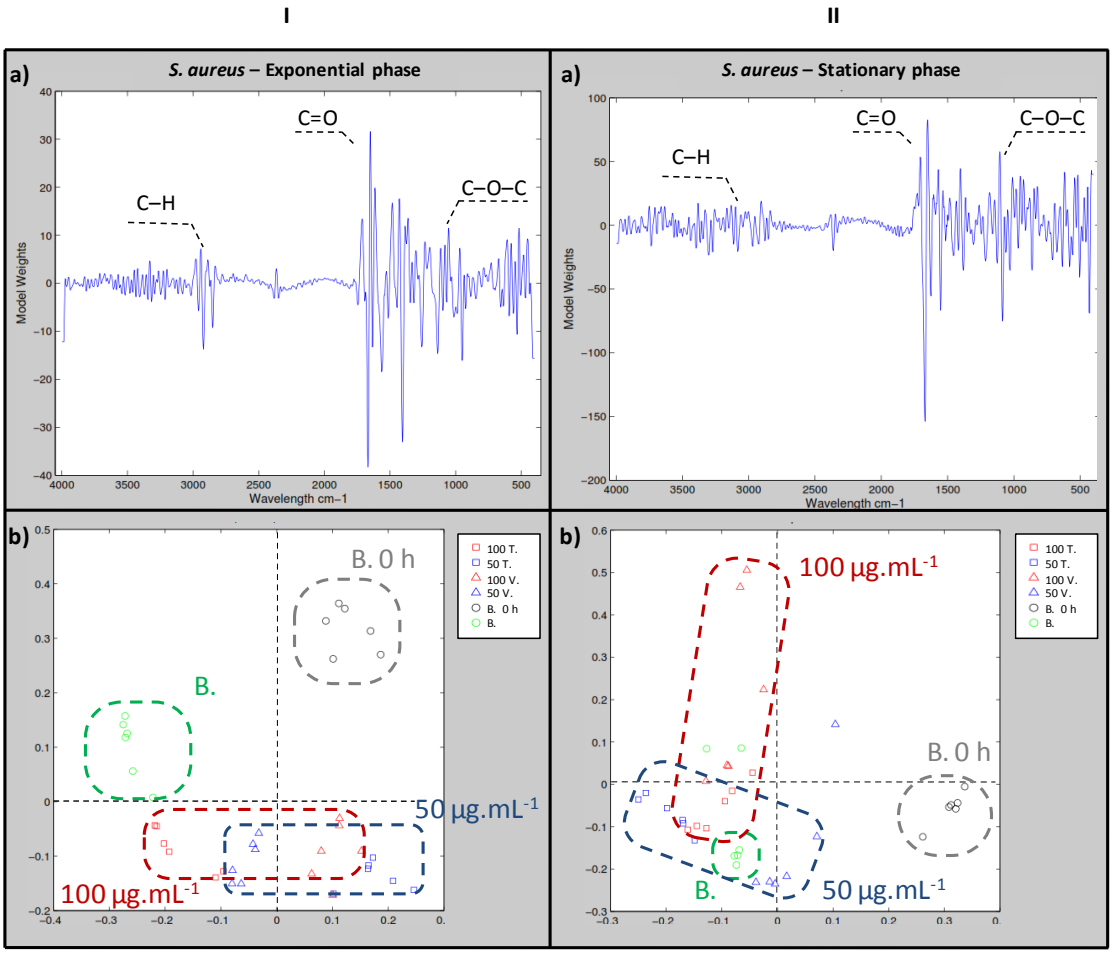

Figure 22 (I) and (II) – FTIR analysis of *S. aureus* cultures after 4 h exposure to high concentrations of vancomycin ('V.') or teicoplanin ('T.'). 'B. 0 h' represents non-exposed cultures at 0 h of culture; 'B.' represents non-exposed cultures at 4 h of culture. **a)** FTIR spectra; **b)** sample distribution in the space formed by the first two principal components determined from principal components analysis (PCA) of the FTIR data. Data acquired and transformed by Bernardo Cunha.

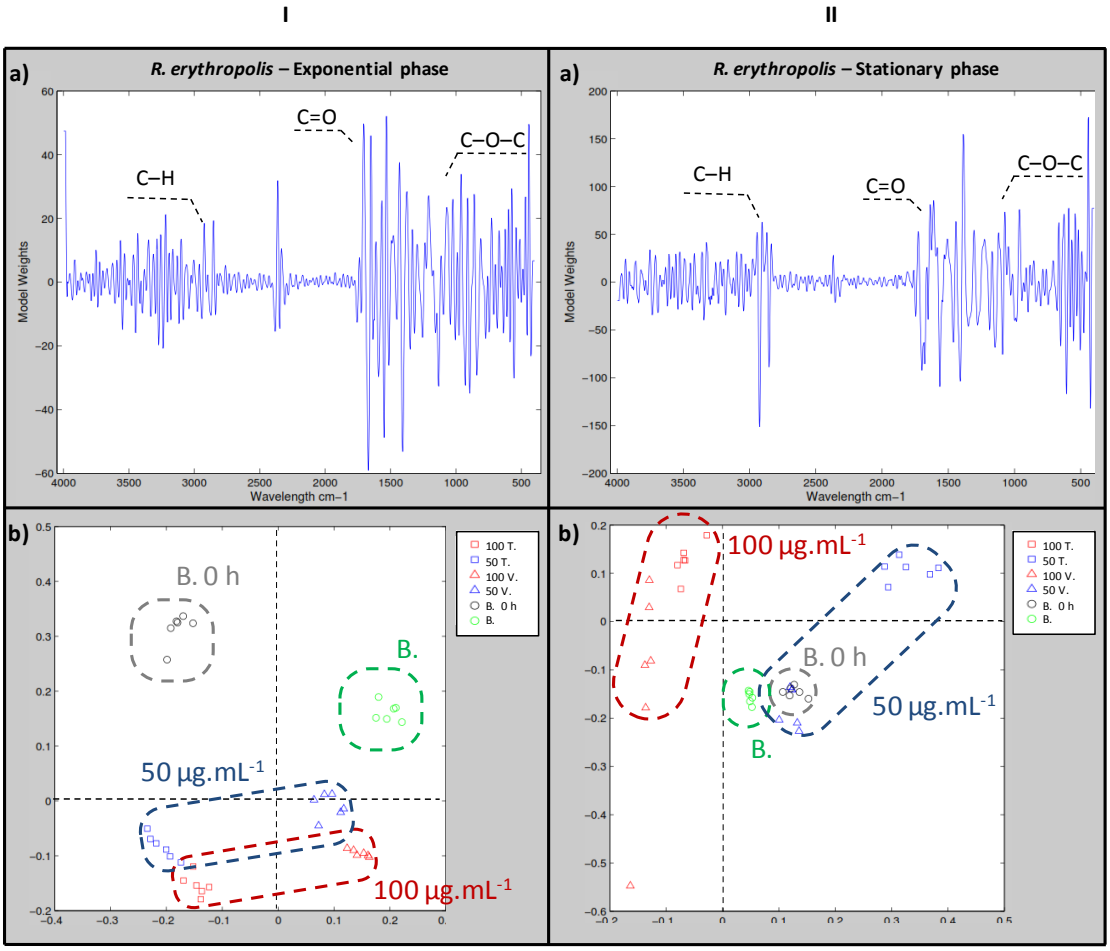

Figure 23 (I) and (II) – FTIR analysis of *R. erythropolis* cultures after 4 h exposure to high concentrations of vancomycin ('V.') or teicoplanin ('T.'). 'B. 0 h' represents non-exposed cultures at 0 h of culture; 'B.' represents non-exposed cultures at 4 h of culture. **a)** FTIR spectra; **b)** sample distribution in the space formed by the first two principal components determined from principal components analysis (PCA) of the FTIR data. Data acquired and transformed by Bernardo Cunha.

The analysis of the registered wavelengths revealed the expected pattern for both bacteria, with the spectral bands characteristic of lipids, C–H, C=O and C–O–C bonds, normally appearing at 2960-2852 cm^{-1}, 1745-1715 cm^{-1}, and 1200-900 cm^{-1} (**Figure 22 (a)** and **Figure 23 (a)**), respectively (Alvarez-Ordóñez et al., 2011). Furthermore, the spectral bands showed an increased intensity in the stationary phase (**Figure 22 II (a)** and **Figure 23 II (a)**), suggesting the expected equilibrium in the formation/degradation of the respective bonds within the bacterial lipids, opposing to the oscillatory behavior that is expected during the exponential phase of growth. A first examination of the PCA distribution results reveals a clear approximation of the different subpopulations with the culture age (from the exponential to the stationary phase of growth) (**Figure 22 (b)** and **Figure 23 (b)**), as expected in the absence of the stress agent, and thus confirming the reversible phenotypic character of persister cells. However, it should be noted that for the highest concentration of antibiotics tested (100 $\mu g.mL^{-1}$) that approximation was not obtained during the time of the experiments, given the more

drastic effects on the exposed populations. The initial blank, 'B. 0 h', showed always a distinct profile, resulting in a clear separation from the other data. Interestingly, a change in the composition of the exposed cells with the age was observed for both strains. During the exponential phase, and regardless of the antibiotic tested, the separation of the data from cells exposed to a concentration of either 50 or 100 $\mu g.mL^{-1}$ was made along PC2 (x axis), while during the stationary phase that separation was made along PC3 (y axis) (**Figure 22 (b)** and **Figure 23 (b)**). These curious results seem to suggest a change at the level of lipid composition and antibiotic response with the age of the culture, as the results from previous sections also indicate. Moreover, cells exposed to a concentration of 50 $\mu g.mL^{-1}$ were represented closer to the non-exposed cells, whilst a concentration of 100 $\mu g.mL^{-1}$ resulted in data further away from non-stressed cells, especially for teicoplanin. These data corroborate the previous observations at the level of *i.* percentage of survived cells, for S*. aureus* and *R. erythropolis* (**Figure 16** and **Figure 17**), and *ii.* cell viability analysis, upon *S. aureus* exposure (**Figure 20**), suggesting a higher potency for teicoplanin when compared to vancomycin.

4.2.5. Lipids Analysis

Apart from the information contained in the genetic 'blueprint', phenotypic modifications constitute a determinant feature of the nature of bacterial adaptive responses as well. Moreover, no matter how similar the genomes of two cells from the same population might be, the response mechanisms of these bacterial cells may be different, which means that in a population not all of the 10^n cells will exhibit identical regulatory transitions (Ryall et al., 2012). When in the presence of a given environmental stress, bacterial cells will adopt particular adaptation mechanisms. These mechanisms have been shown to involve very different changes in either the structure or the composition of the bacterial cells, and may include: i) alterations of the FA composition of the cellular membrane (de Carvalho, 2012; Murínová and Dercová, 2013); ii) modifications of the physicochemical properties of cell surface (*e.g.*, membrane potential; (de Carvalho et al., 2009)); and iii) action of efflux pumps (De Carvalho et al., 2014; Viveiros et al., 2010).

It is now known that the alterations in the FA composition of membrane phospholipids are an important part of the bacterial adaptation mechanisms during exposure to diverse environmental stresses (de Carvalho, 2012; Heipieper and Debont, 1994; Murínová and Dercová, 2013). In fact, lipids are one of the few classes of molecules that may be adjusted in response to environmental stresses by changes in both structure and relative amount, which are essential to maintain the integrity and functionality of the membrane (de Carvalho, 2012). Therefore, a number of studies have been performed with the purpose of determining the effects of cell exposure to different toxic compounds (*e.g.*, pollutants, biocides, fertilizers, antibiotics), under specific environmental, nutritional and/or growth phase conditions, on cell membrane fatty acids (de Carvalho, 2012; de Carvalho et al., 2009; Fernandes et al., 2003; Heipieper and Debont, 1994; Murínová and Dercová, 2013). In these cell lipid composition studies, cell adaptation ability is evaluated by analyzing the differences in FA profile in the different bacterial lipid fractions before and after the exposure, including degree of saturation, *iso*:*ante*-

iso ratio of saturated FA, and changes in each of the main classes of FA (*e.g.*, branched FA). Moreover, cell membrane FA adaptations also depend on the specific stress (or compound concentration) and on the duration of the exposure, as well as on some additional factors, such as the complexity of the strain (Murínová and Dercová, 2013). Therefore, in order to study the adaptation mechanisms of both *S. aureus* and *R. erythropolis* during different times of exposure to each one of the tested antibiotics, cells were collected at specific times during the time-dependent killing curve (**Figure 16** and **Figure 17**, respectively) and the variations at the level of the membrane FA composition were analyzed (**Figure 24** and **Figure 25**, respectively). The branched/saturated FA (**Figure 26 (I)** and **Figure 27 (I)**) and iso/*ante-iso* FA (**Figure 26 (II)** and **Figure 27 (II)**) ratios were also determined from the FA composition obtained for both bacteria.

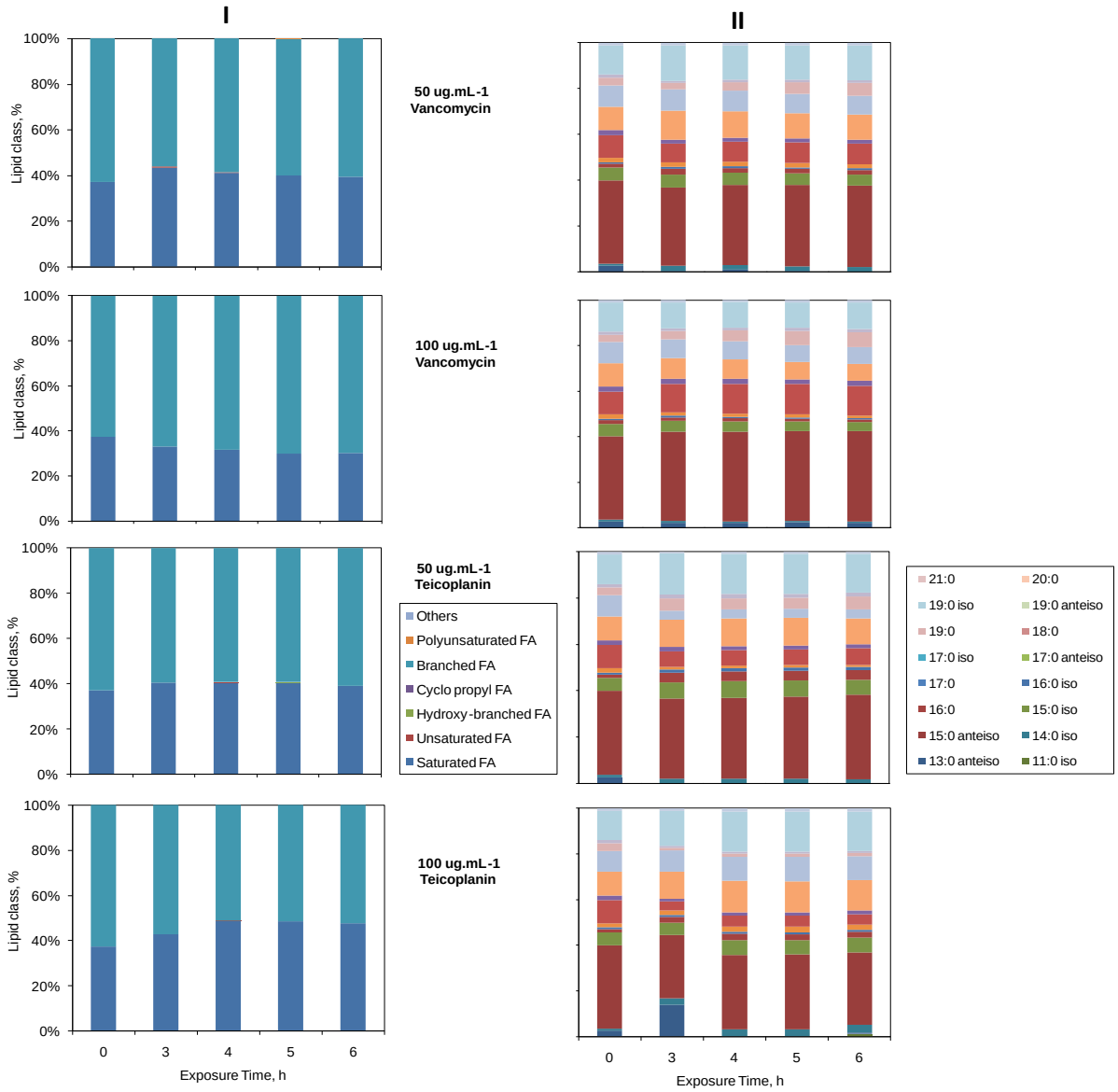

Figure 24 (I) and (II) – Effect of antibiotic concentration and time of exposure on *S. aureus* cultures **(I)** at the level of the composition of the main lipid classes and **(II)** at the level of their FA composition (only those present at concentrations higher than 1% are shown).

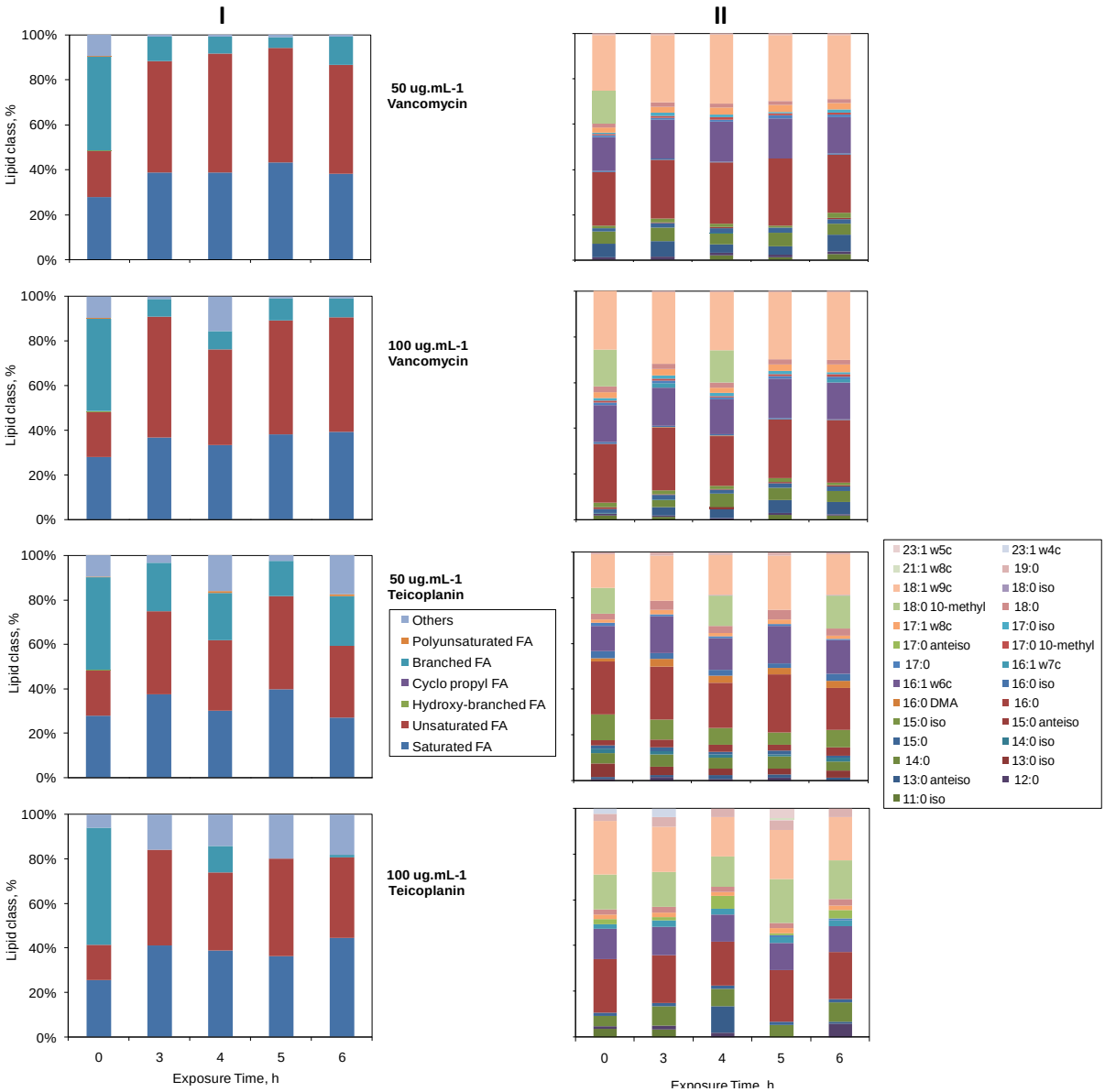

Figure 25 (I) and (II) – Effect of antibiotic concentration and time of exposure on *R. erythropolis* cultures **(I)** at the level of the composition of the main lipid classes and **(II)** at the level of their FA composition (only those present at concentrations higher than 1% are shown).

Both *S. aureus* and *R. erythropolis* cells increased the amount of saturated FA while decreasing the percentage of branched FA (**Figure 24 (I)** and **Figure 25 (I))**, exception being observed for *S. aureus* exposed to 100 µg.mL^{-1} vancomycin (**Figure 24 (I)**). Moreover, most of the alterations occurred during the first 3 h of antibiotic exposure, indicating relatively fast bacterial mechanisms of response. In *R. erythropolis* cells, the percentage of unsaturated FA increased as that of branched FA decreased (**Figure 25 (I)**). Saturated cyclopropyl and hydroxy-substituted FA were observed at concentrations lower than 5% in the collected samples of both bacteria; unsaturated FA were also present at very low amounts in *S. aureus* samples (**Figure 24 (I)**). Furthermore, the analysis of the values presented in **Figure 24 (II)** and **Figure 25 (II)**, shows that the more significant decreases were observed in the

levels of branched FA such as 13:0 *anteiso* (decreased from 2.39 to 0%), 15:0 *anteiso* (from 36.06 to 30.97%), 15:0 *iso* (from 5.46 to 4%) and 17:0 *anteiso* (from 9.83 to 4.46%) in the case of *S. aureus* **Figure 24 (II)**; and 11:0 *iso* (upon 100 µg.mL^{-1} teicoplanin exposure) (from 3.70 to 0%), 13:0 *iso* (from 5.78 to 3.15%) and 15:0 *iso* (from 11.01 to 7.33%) both upon teicoplanin exposure in the case of *R. erythropolis* (**Figure 25 (II)**). On the other hand, the more significant increases were observed in the levels of saturated FA such as 16:0 (upon teicoplanin exposure) (increase from 1.55 to 4.01%), 18:0 (from 10.18 to 13.21%) and 20:0 (from 12.62 to 16.92%) in the case of *S. aureus* (**Figure 24 (II)**); and 14:0 (from 4.59 to 8.52%), 18:0 (from 2.23 to 2.90%), 18:0 10-methyl (from 11.12 to 13.92%) and finally 19:0 (from 2.97 to 3.88%) upon *R. erythropolis* exposure. According to what was seen before for both the percentage of survived cells and the death rates data, the results obtained from FAMEs analysis point towards enhanced mechanisms of response against the presence of antibiotic (in this specific case, vancomycin) in *R. erythropolis*.

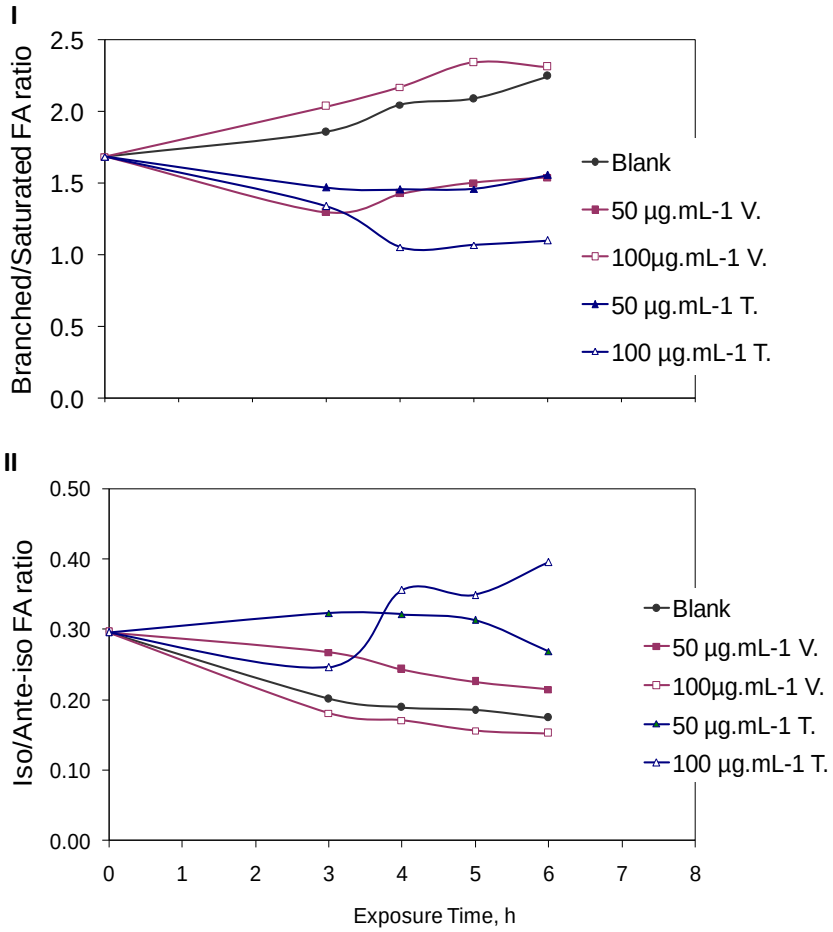

Figure 26 (I) and (II) – Effect of vancomycin ('V.'; squares) and teicoplanin ('T.'; triangles) concentration and time of exposure on *S. aureus* cultures **(I**) at the level of branched/saturated FA and **(II)** iso/*ante-iso* FA ratios. The 'Blank' curves (circles) represent non-exposed cultures.

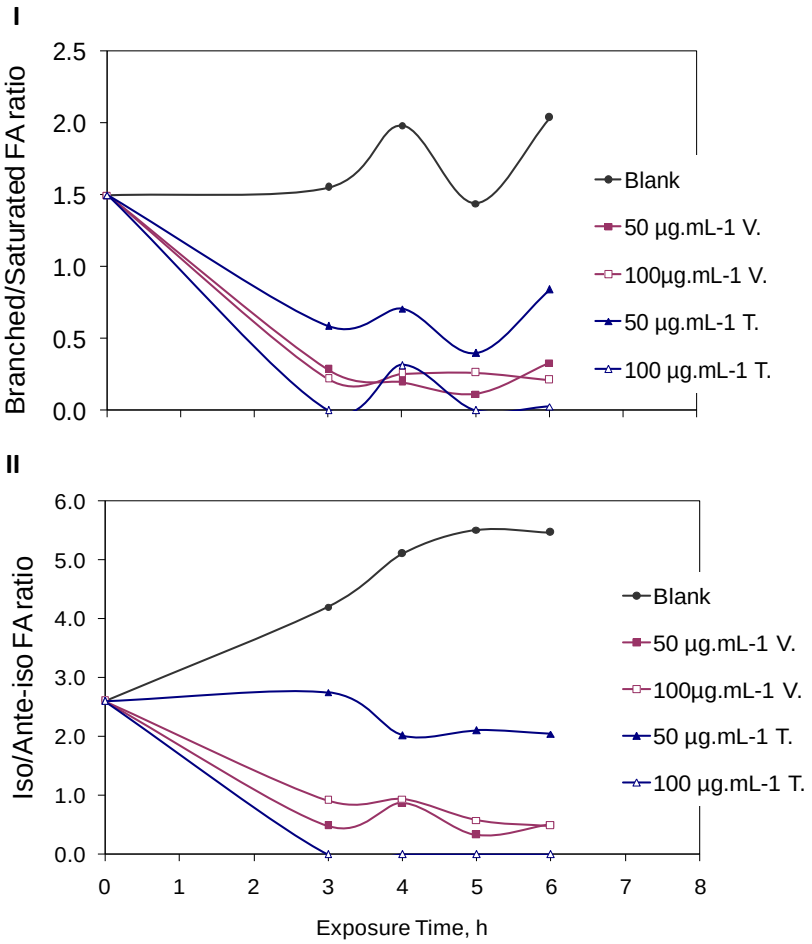

Figure 27 (I) and (II) – Effect of vancomycin ('V.'; squares) and teicoplanin ('T.'; triangles) concentration and time of exposure on *R. erythropolis* cultures **(I)** at the level of branched/saturated FA and **(II)** iso/*ante-iso* FA ratios. The 'Blank' curves (circles) represent non-exposed cultures.

Non-stressed cells of both strains increased the percentage of branched FA while decreasing the percentage of saturated FA with culture age ('Blank', **Figure 10 (II)** and **Figure 11 (II)**). During antibiotic exposure both *S. aureus* and *R. erythropolis* promoted a decrease in the branched/saturated FA ratio (**Figure 26 (I)** and **Figure 27 (I)**, respectively); except for *S. aureus* exposed to 100 $\mu g.mL^{-1}$ vancomycin. Moreover, and according to what was previously observed (**Figure 24** and **Figure 25**), most of the alterations occurred during the first 3 h of antibiotic exposure, indicating relatively fast mechanisms of response. These results strongly suggest that both bacteria respond to vancomycin and teicoplanin induced stress by promoting changes resulting in lower membrane fluidity, thereby confirming what has been generally reported in the presence of toxic organic compounds such as phenol, PAHs, organic solvents and other extreme environmental conditions (de Carvalho, 2012; Heipieper and Debont, 1994; Murínová and Dercová, 2013). Importantly, the oscillatory behavior observed for *R. erythropolis* reveal an oscillatory action of the antibiotics, which can be explained by the fact the tested antibiotics only act on dividing cells.

4.2.6. Zeta Potential Analysis

Bacterial susceptibility to antibiotics has also been studied at the level of the net surface charge of the bacterial envelope (Bishop, 2014; Collins et al., 2002; Peschel et al., 1999). In Peschel *et al.* studies, *S. aureus* and *S. xylosus* were mutated in an attempt to identify genes responsible for their higher tolerance to several antimicrobial peptides (Peschel et al., 1999). The authors obtained highly susceptible mutants, with a modified structure of teichoic acids, lacking D-alanine. As a result, cells increased their negative surface charge, repulsing negatively charged antimicrobial peptides. These results led the authors to propose a role of the D-alanine-esterified teichoic acids, present in several pathogenic bacteria, in the mechanisms of response of those bacteria against antimicrobial (and host defense) agents. A few years later, Collins *et al.* reported that the esterification of specific components of the *S. aureus* cell envelope with amino acids results in the decrease of its net negative surface charge (Collins et al., 2002). Accordingly, *S. aureus* cells started to repulse positively charged antimicrobial peptides. Furthermore, the lack of D-alanine, as shown before, further increased the susceptibility of *S. aureus* to positively charged vancomycin (positive net charge at neutral pH) (Gübitz and Schmid, 2004) and other glycopeptides. Similar results were recently obtained by Jia et al. (Jia et al., 2013). Alternatively, Bishop R.E. most recent results, suggest that the production of anionic non-bilayer lipids likely increases the tolerance of Gram-negative bacteria outer membranes against antibiotics with structures similar to those of CAMPs and vancomycin (Bishop, 2014).

In the present study, an overall decrease in the ZP values of the cells was observed with both concentration and time of exposure for both bacterial species studied (**Figure 28 (I)** and **(II)**). A greater response was obtained upon exposure to 100 $\mu g.mL^{-1}$ teicoplanin, with membrane ZP decreasing from -14.8 to -25 mV after a total of 6 h of exposure in the case of *S. aureus* (**Figure 28 (I)**) and from -24.9 to -36.1 mV after the same period of exposure in the case of *R. erythropolis* (**Figure 28 (II)**). Similarly to what was observed at the level of lipid composition (**Figure 24** to **Figure 27**), the most significant changes occurred during the first 3 h of antibiotic exposure. The observed increase in the cell wall negative net surface charge as a response mechanism against teicoplanin is expected, since this compound is negatively charged at neutral pH (Gübitz and Schmid, 2004). Therefore, such an increase in the negative character of the cells will result in the repulsion of teicoplanin. On the other hand, the same principle is not valid for the positively charged vancomycin (Gübitz and Schmid, 2004), which would that way be attracted to a more negative membrane. However, the behavior observed for vancomycin exposure seems to corroborate the most recent advances on the subject (Bishop, 2014; Cafiso et al., 2012).

Moreover, and after equivalent results obtained both at the levels of percentage of survived cells (**Figure 17**) and membrane FA composition (**Figure 24** to **Figure 27**) suggested the same, now the increased responses at the level of membrane ZP profile (relatively to those of *S. aureus*) point, as well, towards an enhanced antibiotic tolerance for *R. erythropolis*.

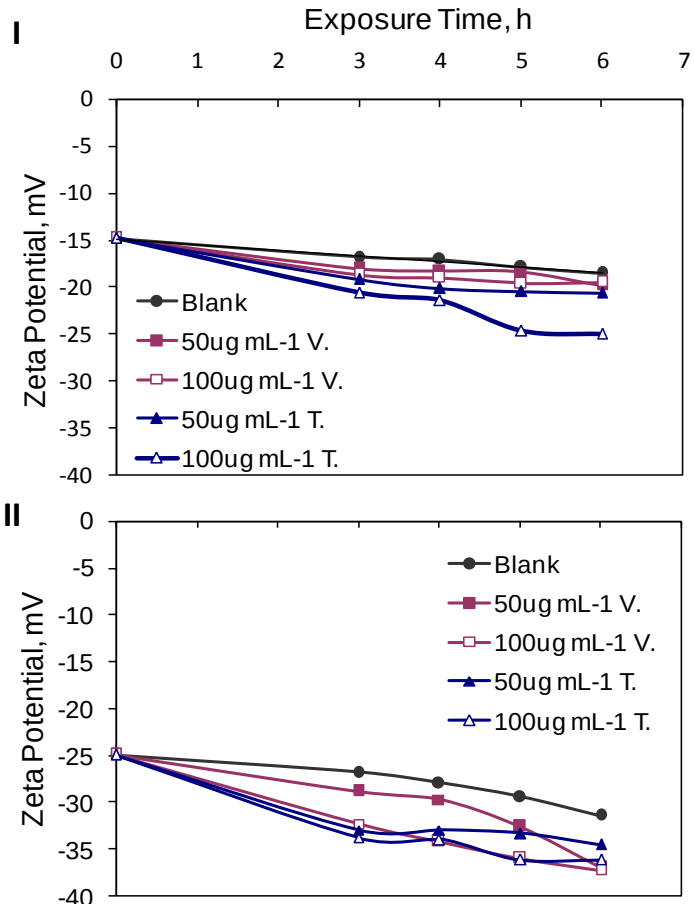

Figure 28 (I) and (II) – Effect of vancomycin ('V.'; squares) and teicoplanin ('T.'; triangles) concentration and time of exposure on the zeta potential of *S. aureus* **(I)** and *R. erythropolis* **(II)** cells. The 'Blank' (circles) curve represents non-exposed culture.

4.2.7. Oxygen Consumption Analysis

To determine the metabolic activity of the cells exposed to each antibiotic/concentration, the oxygen consumption of each culture was monitored. To test the effect of low antibiotic concentrations, *S. aureus* cells were exposed for 16 h to vancomycin (**Figure 29 (I)**) and for 20 h to teicoplanin (**Figure 29 (II)**) at the following concentrations: 2, 3, 4.6, 6, 9, 12.5, 18, 25, 37.5, 50, 75 and 100 $\mu g.mL^{-1}$. To test the effect of high antibiotic concentrations, both *S. aureus* (**Figure 31 (I)**) and *R. erythropolis* (**Figure 31 (II)**) were exposed to each antibiotic at a concentration of 50 or 100 $\mu g.mL^{-1}$ for a total of 31 and 29 h, respectively. The respective oxygen consumption rates were also determined.

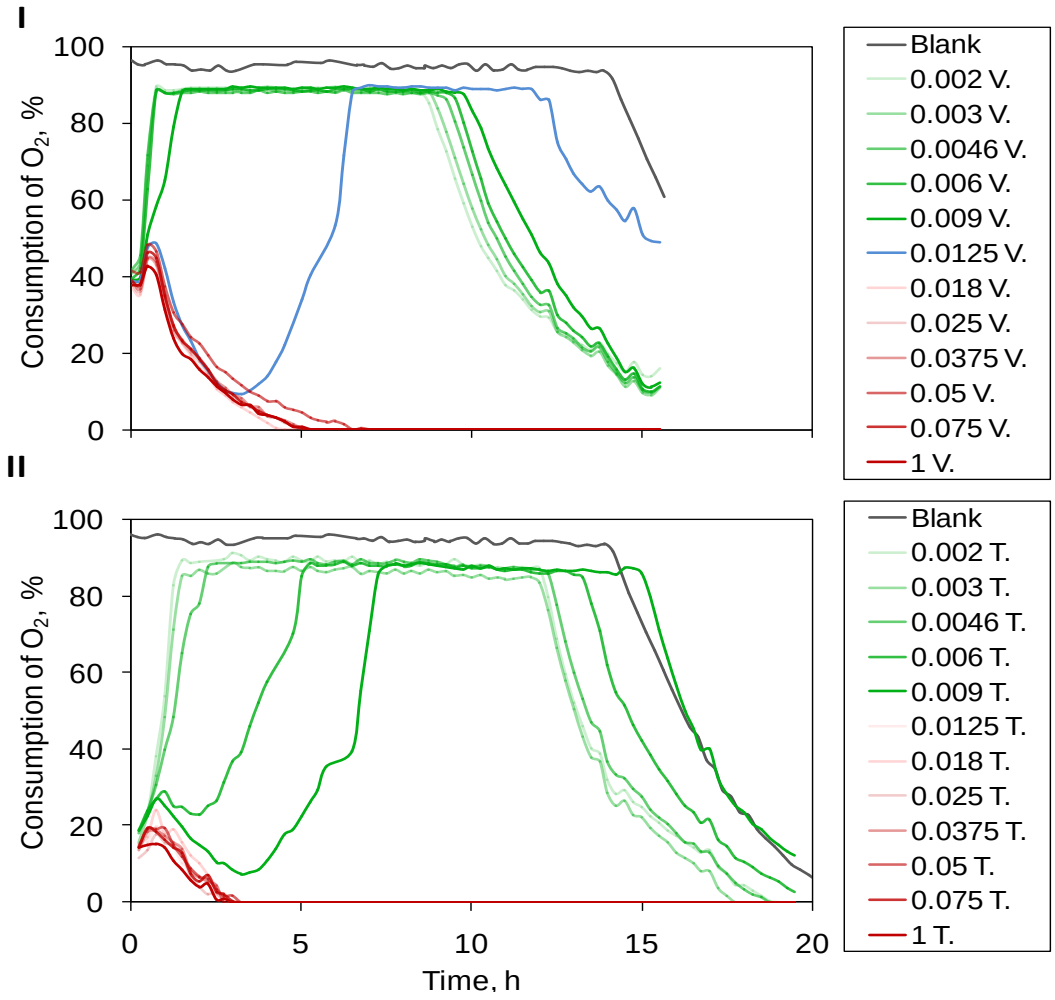

Figure 29 (I) and (II) – Effect of vancomycin ('V.') **(I)** and teicoplanin ('T.') **(II)** concentrations on *S. aureus* oxygen consumption (at low antibiotic concentration). The 'Blank' curves represent non-exposed cultures.

The oxygen consumption curves at low antibiotic concentrations indicate, as expected and for both antibiotics, that the consume decreases with the increasing antibiotic concentrations (**Figure 29 (I)**). Moreover, three distinct profiles were observed (for 12.5 $\mu g.mL^{-1}$, and for concentrations below and above this value). At conditions lower than 12.5 $\mu g.mL^{-1}$, the cells managed to consume high amounts of oxygen during the first 10-15 h, after which levels successively lowered, until no oxygen was consumed, similarly to what was observed in the absence of antibiotics ('Blank' curve, **Figure 29**). On the contrary, for antibiotic concentrations higher than 12.5 $\mu g.mL^{-1}$ a distinct behavior was obtained, suggesting an immediate effect on cells' metabolic activity, at least at the level of oxygen consumption. For those concentrations, the cells decreased considerably their levels of oxygen requirements during the first hour, with the consumption ceasing before 5 h of exposure. Together, the results suggest that antibiotic concentrations as low as 12.5 $\mu g.mL^{-1}$ are enough to trigger changes with repercussion in the metabolic activity of the cells, as observed by oxygen consumption.

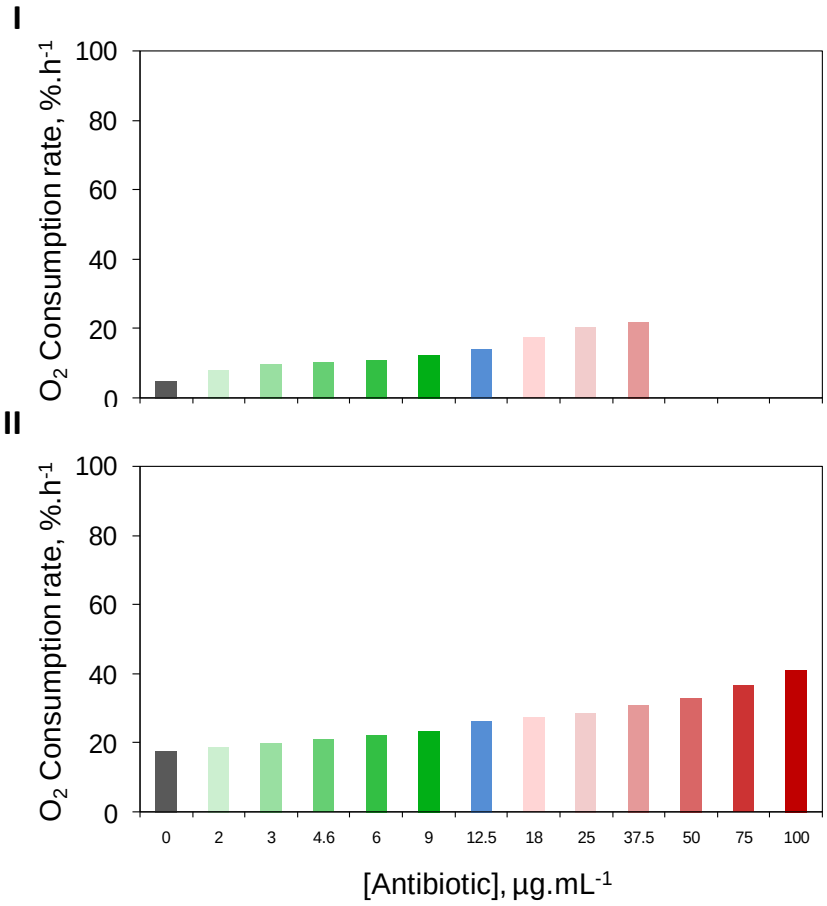

Figure 30 (I) and (II) – *S. aureus* oxygen consumption rates during vancomycin ('V.') **(I)** and teicoplanin ('T.') **(II)** exposure (at low antibiotic concentration). The 'Blank' bar represents non-exposed culture. Oxygen consumption rates were calculated using the values within the interval of oxygen consumption decay (**Figure 29**).

The oxygen consumption rates increased with increasing antibiotic concentration (**Figure 30**). When exposed to the antibiotic, the cells must switch-on a series of oxygen-requiring adaptive mechanisms to counteract the stress caused. Orman and Brynildsen have also shown that even cells in a non-growing state can be metabolically active (Orman and Brynildsen, 2013).

The results ultimately indicate that, instead of entering in a 'dormant', inactive state, persister cells are, despite their expected non- or slow-growing phenotype, in a truly active response state. This has also been recently defended by other authors (Orman and Brynildsen, 2013; Wakamoto et al., 2013).

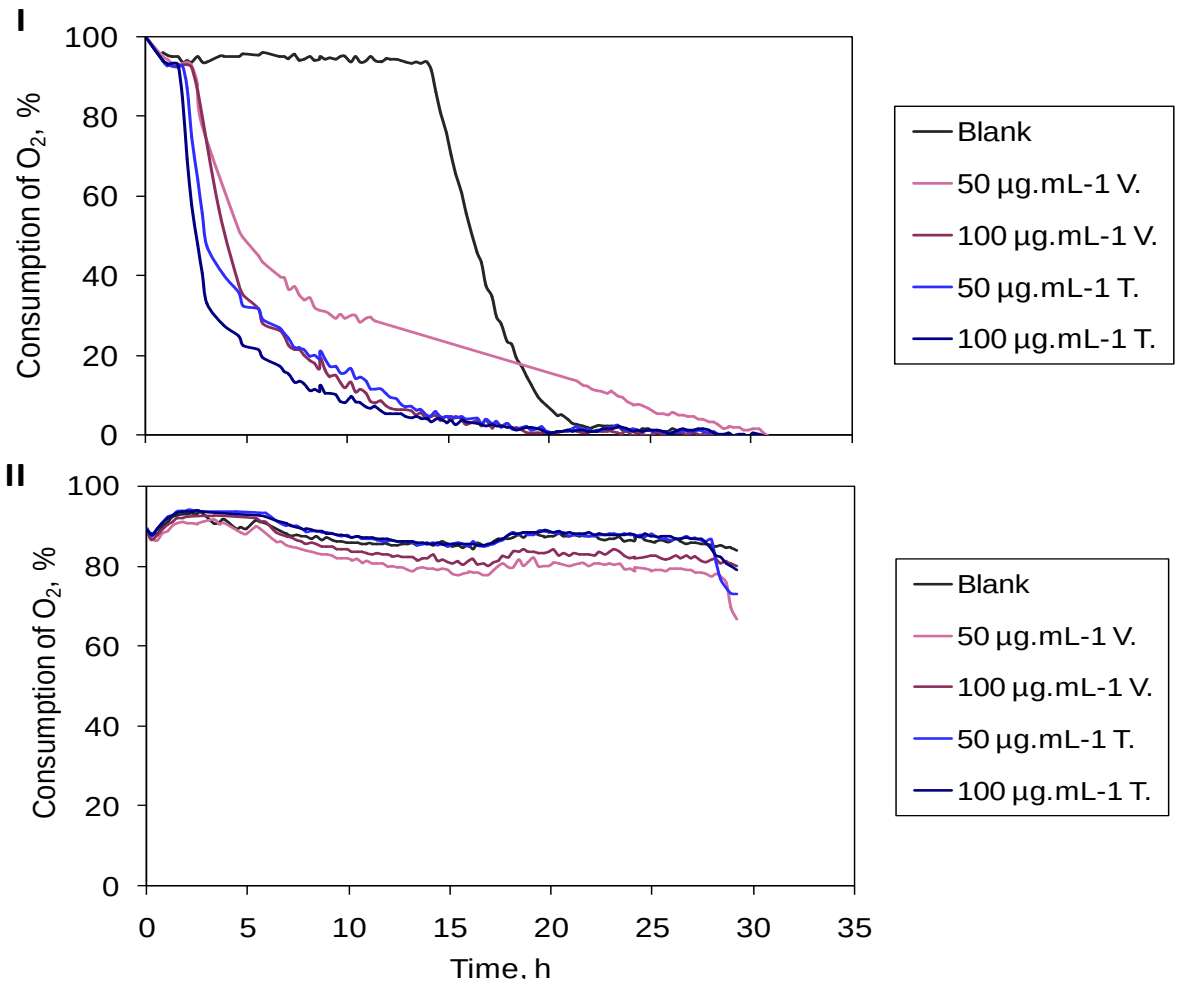

Figure 31 (I) and **(II)** – Effect of vancomycin ('V.') and teicoplanin ('T.') concentrations on *S. aureus* **(I)** and *R. erythropolis* **(II)** oxygen consumption (at high antibiotic concentration). The 'Blank' curves represent non-exposed cultures.

Oxygen consumption by *S. aureus* cells in the presence of high antibiotic concentrations decreased with the increasing antibiotic concentration (**Figure 31**). After 2-3 h of exposure, the cells decreased the level of oxygen consumption, this time with the consume ceasing around 20 h of exposure. Nevertheless, and although the oxygen consumption decreased once more with the antibiotic concentration, the results reveal an overall increase relatively to that obtained at low antibiotic concentrations (**Figure 29**), suggesting an active response to such elevated concentrations. The oxygen consumption rates obtained *S. aureus* exposed to such high antibiotic concentrations (**Erro! A origem da referência não foi encontrada. (I)**), suggest, as before, that the cells actively respond to the increase in antibiotics concentration, especially in the case of teicoplanin.

In the case of *R. erythropolis*, the opposite evolution was observed: both the blank and challenged cells maintained the levels of oxygen consumption, for almost 30 h of exposure, at around 80-100% regardless of the antibiotic/concentration tested. Once again, the increased metabolic activity. *i.e.* increased levels of oxygen consumption, experienced by the non- or slow-growing persisters corroborates the recent results of Orman and Brynildsen (Orman and Brynildsen, 2013). This behavior is also in accordance with the results obtained *i.* at the level of percentage of survived cells (**Figure 17**); *ii.* for membrane FA composition (**Figure 25** and **Figure 27**); and *iii.* for cell surface charge (**Figure 28 (II)**); which together strongly suggest an enhanced antibiotic tolerance for *R. erythropolis*. This tolerance is thus likely achieved thanks to a more active metabolic activity (**Erro! A origem da referência não foi encontrada. (II)**).

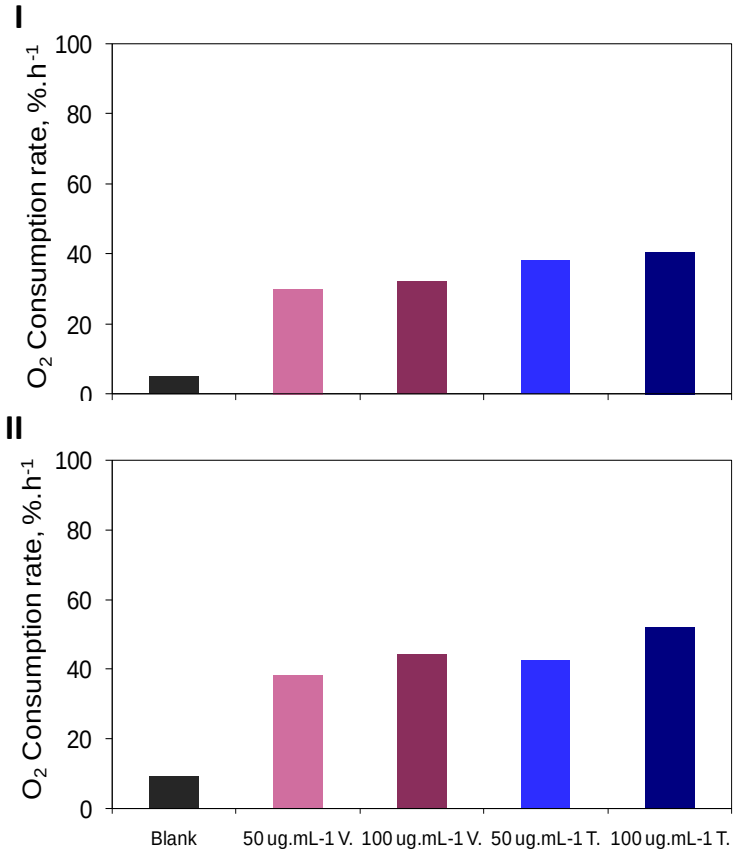

Figure 32 (I) and (II) – *S. aureus* **(I)** and *R. erythropolis* **(II)** oxygen consumption rate during vancomycin ('V.') and teicoplanin ('T.') exposure (at high antibiotic concentration). The 'Blank' bars represent non-exposed cultures. Oxygen consumption rates were calculated using the values within the interval of oxygen consumption decay (**Figure 31**).

4.2.8. Enzymatic Activity Analysis

The metabolic activity of the cells was also accessed by comparison of the enzyme activity of 11 enzymes present in the API ZYM® kit (**Figure 33**).

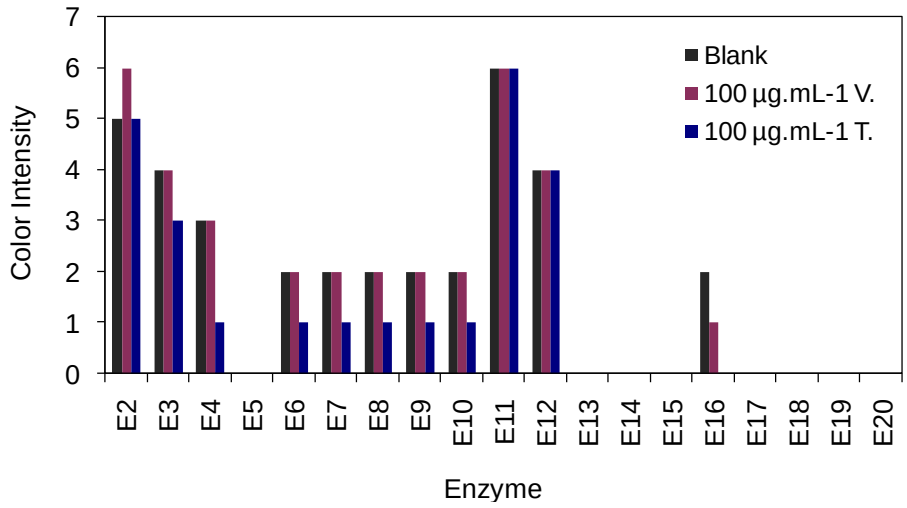

Figure 33 – Enzymatic activity of 11 enzymes in *S. aureus* prior and after 6 h of exposure to high vancomycin ('V.') or teicoplanin ('T.') concentrations. The 'Blank' bars represent non-exposed cultures. Color intensity: 0 (no activity) to 6 (strong activity). The horizontal axis corresponds to the following enzymes: E2, alkaline phosphatase; E3, esterase (C4)**;** E4, esterase lipase (C8)**;** E5, lipase (C14); E6, leucine arylamidase; E7, valine arylamidase; E8, cystine arylamidase; E9, trypsin; E10, α-chymotrypsin; E11, acid phosphatase; E12, Naphtol-AS-BI-phosphohydrolase; E1**3,** α-galactosidase; E14, β-galactosidase: E15, β-glucuronidase; E16, α-glucosidase; E17, β-glucosidase; E1**8,** N-acetyl-β-glucosaminidase; E19, α-mannosidase; E20, α-fucosidase.

A first analysis of the obtained enzyme profiles reveals that, even upon exposure, the survivor, persister cells managed to maintain unchanged the activity of the majority of the prior active enzymes, corroborating the results obtained at the level of oxygen consumption (vide section **4.2.7. Oxygen Consumption Analysis**) and thus refuting the previous theories claiming that this class of cells enters a 'dormant', inactive state. However, a closer analysis shows that, after teicoplanin exposure, the activity of some enzymes such as esterase (C4) (E3), esterase lipase (C8) (E4), leucine, valine and cystine arylamidase (E6 to E8), trypsin (E9) and α-chymotrypsin (E10) were slightly reduced as a direct effect of the presence of the antibiotics. A reduced enzymatic activity was also observed for α-glucosidase (E16) after exposure to vancomycin, with no considerable activity being reported after

exposure to teicoplanin. The specific function of each enzyme will be discussed in section **4.3.6. Enzymatic Activity Analysis**.

4.3. Bacterial Re-Growth in Fresh Medium (after Stress Conditions)

4.3.1. Effect of the Age of the Culture on the Normal Growth

As mentioned, it was expected that the (re-)growth of persister cells of both *S. aureus* and *R. erythropolis* followed the non-exposed cultures ('Blank') when placed in 'fresh' MHB. It was also necessary to assure that the non-stressed control cells could produce similar growth curves when placed again in fresh medium, *i.e.*, that no age-controlled effect could influence the growth curve of the cells. The results showed that the growth of both the 'Blank' cells and those that had remained for 3 h in MHB before being used to re-inoculate fresh MH medium followed the same evolution pattern, differing only at the final stages of growth (**Figure 34 (I)** and **(II)**). Similar duplication times were obtained for each bacteria under both conditions: 0.71 h^{-1} for the 'Blank' and 1.66 and 2.03 h^{-1} for the duplicates of 'Re-Growth' cells in the case of *S. aureus*; whilst 0.85 h^{-1} was obtained for the 'Blank' and 0.68 and 0.70 h^{-1} for the 'Re-Growth' in the case of *R. erythropolis* cells. The results thus support that a 'Blank', non-exposed culture can, efficiently, be used as a control during the re-growth of the exposed cultures (after drug removal).

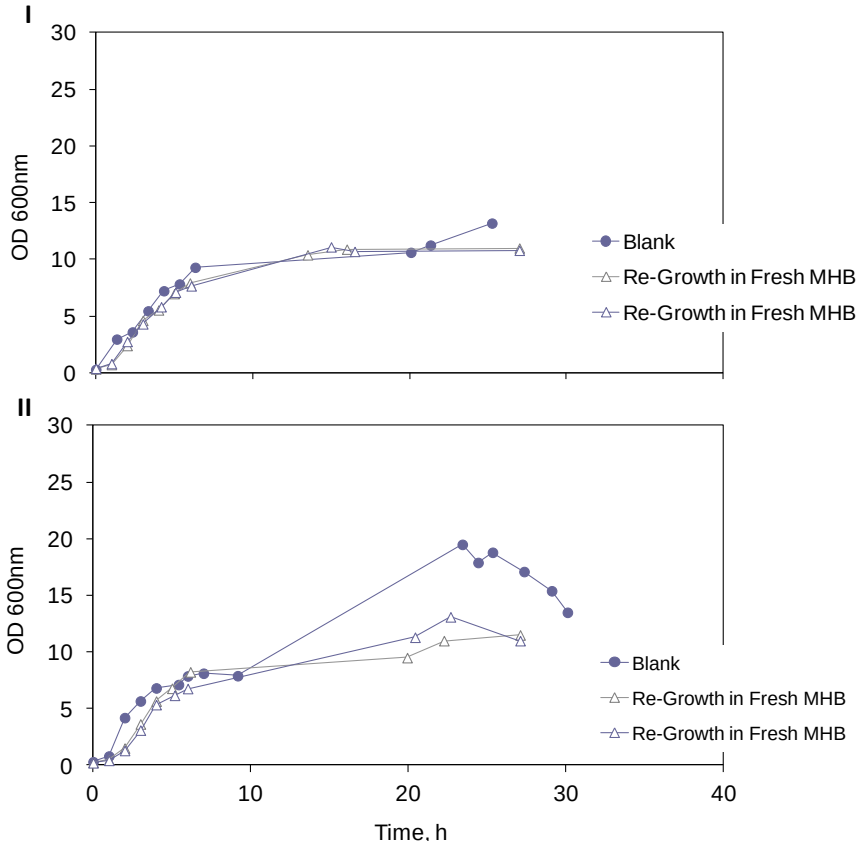

Figure 34 (I) and (II) – *S. aureus* **(I)** and *R. erythropolis* **(II)** growth curves in MHB: regular growth ('Blank', circles); and after placing a 3 h culture in fresh MHB (duplicates of 'Re-Growth in Fresh MHB', triangles).

4.3.2. Repopulation of the Culture after Drug Removal

Contrarily to what is observed during a normal growth (**Figure 35 a)**), the large majority of susceptible cells composing a bacterial growing population will, upon antibiotic exposure, be non-viable, killed or even lysed. However, a small subpopulation of highly tolerant cells, persister cells, will be able to survive (**Figure 35 b)**). This small number of persister cells will therefore enrich the original bacterial population and will, when the conditions in the environment improve (*i.e.*, when the antibiotic concentration reaches non-lethal levels), allow the repopulation of the culture (**Figure 35 c)**). If the cells are persister and not resistant cells, the growth curve should follow that of the original, non-stressed population. In the present study, and to assess if the strategy used has been successful in producing persister cells, the cultures of both *S. aureus* and *R. erythropolis* were analyzed in terms of the re-growth and repopulation after antibiotic exposure and subsequent growth in drug-free medium (**Figure 36** and **Figure 37**, respectively).

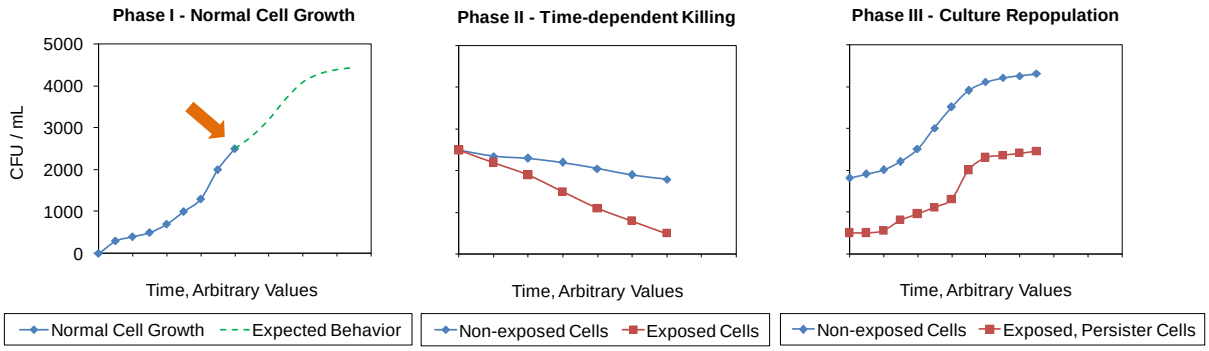

Figure 35 a) to c) – Growth and killing bacterial patterns before, during and after antibiotic exposure. **a)** Normal growth of a bacterial population before (full line) addition of an antibiotic and the expected (dashed line) behavior of the same population in the absence of the antibiotic – Phase I of an antibiotic exposure. The arrow indicates the time at which the antibiotic is added. **b)** Time-dependent killing pattern upon the exposure of the population to a high concentration of an antibiotic, which results in cell death, leaving only persister cells alive (opposed to a non-exposed population) – Phase II of an antibiotic exposure. **c)** Culture repopulation from the survivor persister cells – Phase III of an antibiotic exposure. Adapted from (Lewis, 2007).

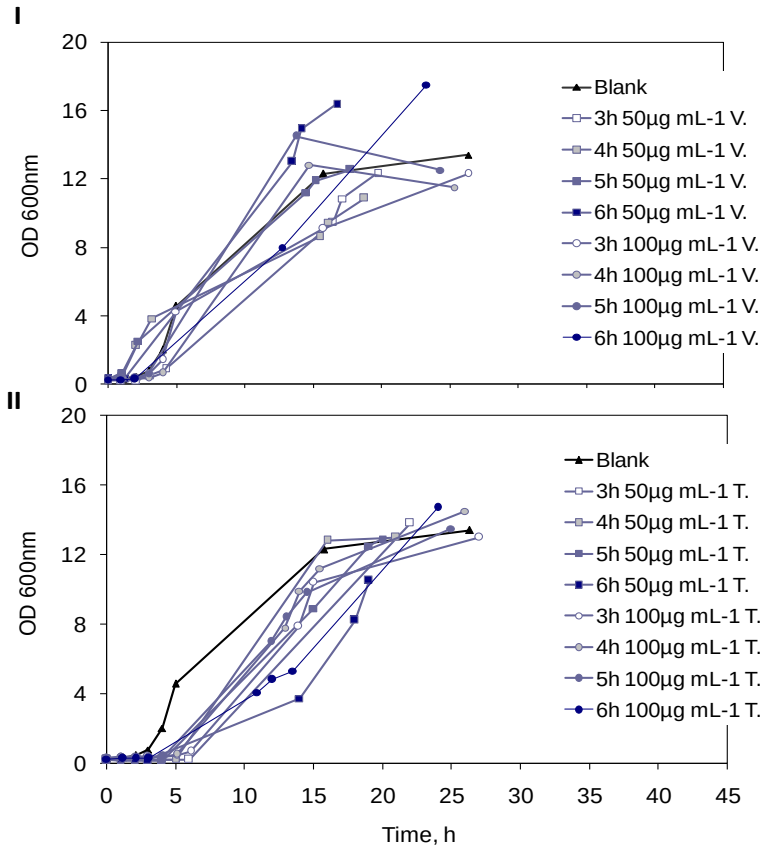

Figure 36 (I) and (II) – Growth in fresh, antibiotic-free medium of *S. aureus* cells that had survive exposure for 6 h to 50 (squares) and 100 µg.mL^{-1} (circles) vancomycin ('V.') **(I)** or teicoplanin (' T.') **(II)**. The 'Blank' curves (triangles) represent non-exposed cultures.

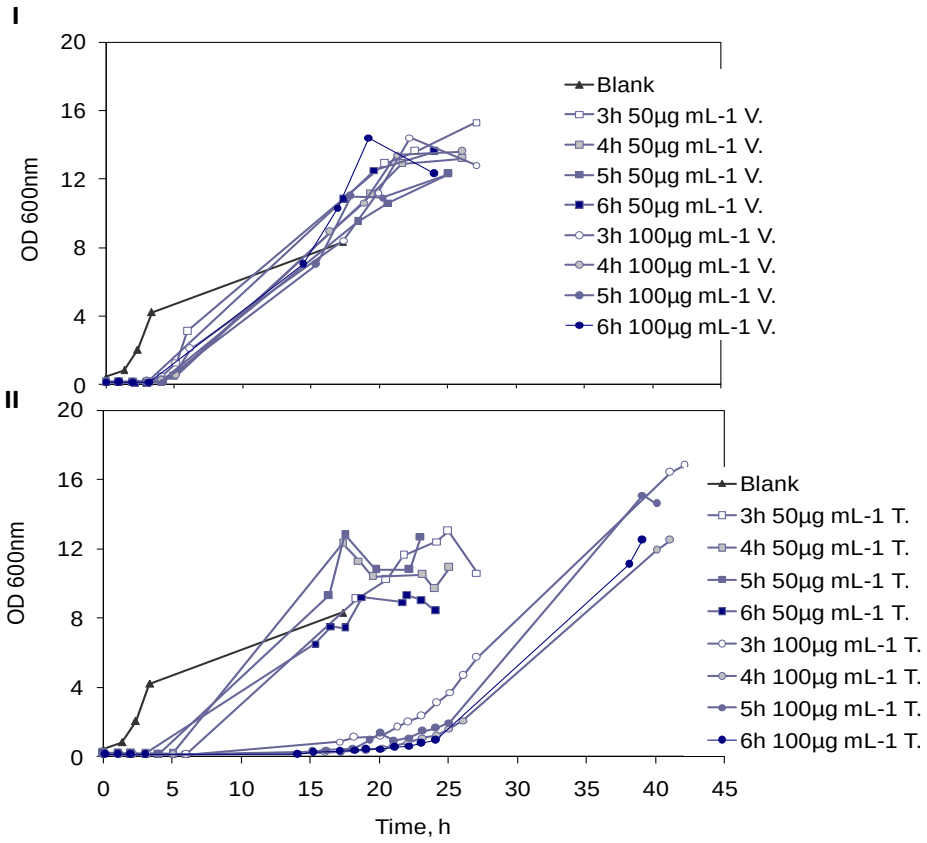

Figure 37 (I) and (II) – Growth in fresh, antibiotic-free medium of *R. erythropolis* cells that had survive exposure for 6 h to 50 (squares) and 100 µg mL^{-1} (circles) vancomycin ('V.') **(I)** or teicoplanin (' T.') **(II)**. The 'Blank' curves (triangles) represent non-exposed cultures.

According to Lechner *et al.* (Lechner et al., 2012), persister tolerance is not transferred to the progeny, therefore, a similar behavior to that observed during normal cell growth (**Figure 35 a)**) is expected during the re-growth of the survivor persister subpopulation in fresh, drug-free medium. The curves represented in **Figure 36** and **Figure 37** show the expected pattern, described by a slow increase in biomass, rather than a fast increase (typical for resistant, mutant cells after acquiring resistance). Therefore, the small subpopulation of cells that managed to survive antibiotic exposure is, most likely, composed of persister cells. The cultures that were in contact with the antibiotic for a longer period of time presented longer *lag* phases (**Figure 16** and **Figure 17**). Here, the results obtained for *R. erythropolis* re-growth after 100 µg.mL^{-1} teicoplanin exposure really stood out from the rest, with cells requiring almost four times the period of time needed for the re-growth of the same bacteria after 50 µg.mL^{-1} exposure (**Figure 37 (II)**). Nevertheless, and for both bacteria, the cultures previously exposed to each antibiotic/concentration presented a similar (re-)growth to that of 'Blank', non-exposed

cultures, thus confirming the persister nature of the subpopulation of survivor cells (**Figure 36** and **Figure 37**).

4.3.3. Lipid Analysis

To assess if lipid composition of the persister cells was more similar to that of non-stressed cells or of those cells exposed to antibiotics, the cells were previously exposed to either low or high antibiotic concentrations, for different periods of time. In the former case, *S. aureus* cells were exposed to both vancomycin and teicoplanin at the following concentrations: 2, 3, 4.6, 6, 9, 12.5, 18, 25, 37.5, 50, 75 and 100 $\mu g.mL^{-1}$ for up to 20 h (**Figure 38** and **Figure 39**, respectively); whilst in the latter case, both *S. aureus* and *R. erythropolis* cells were exposed to each antibiotic at a concentration of 50 or 100 $\mu g.mL^{-1}$ for up to 31 h (**Figure 40** and **Figure 41**; and **Figure 42** and **Figure 43**; respectively).

The cells of *S. aureus* grown on fresh media presented lipid compositions that depend on the concentration at which the previous population had been exposed to (**Figure 38 (I)** and **Figure 39 (I)**). For the lower, less effective concentrations (from 2 to 37.5 $\mu g.mL^{-1}$), the cells FA composition was similar to that of cells prior to exposure: high levels of branched FA and low levels of saturated FA. However, for the higher concentrations tested (75 and 100 $\mu g.mL^{-1}$ in the case of teicoplanin and, additionally, 50 $\mu g.mL^{-1}$ in the case of vancomycin), the cells presented different FA composition: the percentage of saturated straight FA increased, whilst the percentage of branched FA decreased. Comparing the specific values presented in **Figure 38 (II)** and **Figure 39 (II)** for the cells which have restored their composition with those which have not, it is possible to conclude that the more significant changes were observed at the levels of saturated FA such as 12:0 (increase from 0.13 to 6.91%), 14:0 (from 0.29 to 1.86%), 16:0 (from 2.16 to 12.72%) and 18:0 (from 11.54 to 19.58%) in the case of cells pre-exposed to vancomycin (**Figure 38 (II)**); and 19:0 (from 3.14 to 4.46%), 19:0 *anteiso* (from 3.96 to 13.85%) and 20:0 (from 0 to 10.09%) in the case of cells pre-exposed to teicoplanin (**Figure 39 (II)**). On the other hand, the more significant decreases were observed in the levels of branched FA such as 15:0 *anteiso* (decrease from 34.18 to 19.13%), 15:0 *iso* (from 8.78 to 1.36%) and 17:0 *anteiso* (from 10.99 to 6.78%) in the case of cells pre-exposed to vancomycin (**Figure 38 (II)**); and 15:0 *iso* (from 10.28 to 3.32%) and 17:0 *iso* (from 5.17 to 1.65%) in the case of cells pre-exposed to teicoplanin (**Figure 39 (II)**). A higher degree of saturated FA and a lower percentage of branched FA, should have resulted in less fluid membranes, thus these cells should be able to prevent the entrance of recalcitrant compounds to the cell. What is most significant is that these changes in lipid composition were transmitted to a non-stressed population by the parent cells. Contrarily to resistance mechanisms which are encoded in the DNA sequence, phenotypic modifications have a limited life time but the 'memory' of past conditions and stress-induced molecules may influence the future generations (Gefen and Balaban, 2009).

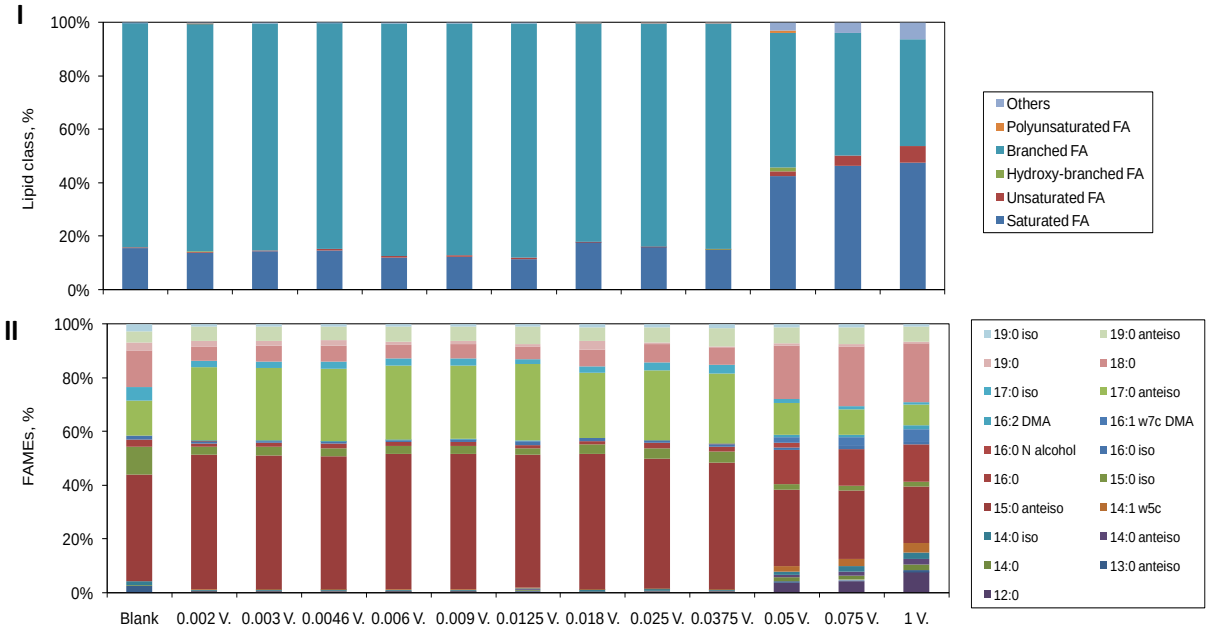

Figure 38 (I) and (II) – Lipid composition of *S. aureus* cells grown in fresh medium without antibiotic for 20 h. The cultures were inoculated with cells exposed to low vancomycin concentrations. **I:** lipid composition in terms of the chemical structure of the FA; **II:** composition in FA (only those present at concentrations higher than 1% are shown). The 'Blank' bars represent non-exposed cultures.

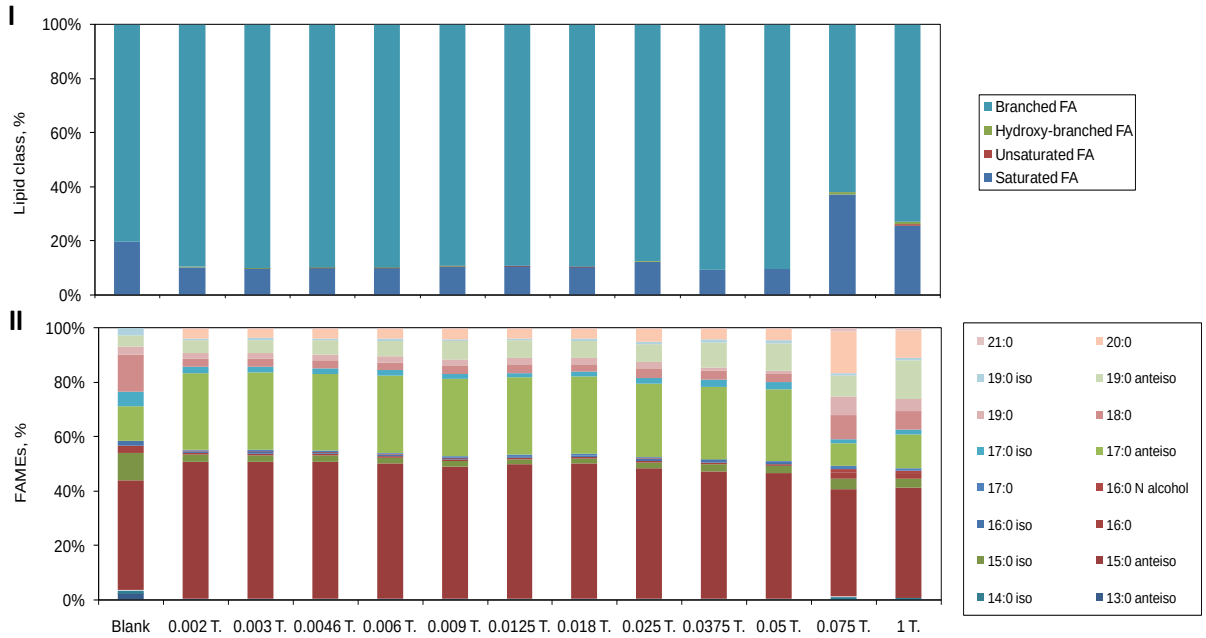

Figure 39 (I) and (II) – Lipid composition of *S. aureus* cells grown in fresh medium without antibiotic for 20 h. The cultures were inoculated with cells exposed to low teicoplanin concentrations. **I:** lipid composition in terms of the chemical structure of the FA; **II:** composition in FA (only those present at concentrations higher than 1% are shown). The 'Blank' bars represent non-exposed cultures.

For the re-growth of the both *S. aureus* and *R. erythropolis* cells pre-exposed to high antibiotic concentrations, the changes registered in the percentages of each class of FA showed that the lipid composition of the cells presented high levels of branched FA and low levels of saturated FA, similar to non-exposed cells (**Figure 10** and **Figure 11**, respectively). Furthermore, the harsher the previous conditions of exposure (*i.e.,* higher antibiotic concentration and/or longer period of exposure), the longer time the cells needed to return to its original composition during the re-growth. Nevertheless, after 24 h of re-growth, the cells generally acquired a composition identical to that of the initial non-exposed population. Furthermore, analyzing the specific values for the FA composition of the cells presented in **Figure 40 (II)** to **Figure 43 (II)**, it is possible to conclude that the more significant changes were observed at the levels of saturated FA such as 12:0 (decrease from 11.93 to 0%), 16:0 (from 26.01 to 1.24%), 18:0 (from 20.63 to 6.43%), 19:0 (from 5.54 to 1.01%) and 20:0 (from 11.28 to 6.17%) in the case of *S. aureus* cells pre-exposed to vancomycin (**Figure 40 (II)**) and 12:0 (from 23.48 to 0.12%), 16:0 (from 13.28 to 1.38%), 18:0 (from 28.30 to 4.80%) and 20:0 (from 10.12 to 5.86%) in the case of *S. aureus* cells pre-exposed to teicoplanin (**Figure 41 (II)**); and 12:0 (from 7.05 to 0.21%), 16:0 (from 13.39 to 6.71%) and 18:0 (from 6.37 to 1.01%) in the case of *R. erythropolis* pre-exposed to vancomycin (**Figure 42 (II)**) and 12:0 (from 19.01 to 0.86%), 14:0 (from 9.43 to 1.56%), 16:0 (from 31.75 to 24.82%) and 18:0 (from 19.53 to 0%) in the case of *R. erythropolis* pre-exposed to teicoplanin (**Figure 43 (II)**). On the other hand, the more significant increases were observed in the levels of branched FA such as 13:0 *anteiso* (increase from 4.89 to 20.70%), 15:0 *anteiso* (from 7.57 to 34%), 15:0 *iso* (from 0 to 3.45%), 17:0 *anteiso* (from 1.56 to 18.12%) in the case of *S. aureus* cells pre-exposed to vancomycin (**Figure 40 (II)**) and 15:0 *anteiso* (from 27.03 to 41.42%), 17:0 *anteiso* (from 0 to 20.14%), 17:0 *iso* (from 0 to 3.99%) and 19:0 *anteiso* (from 0 to 6.94%) in the case of *S. aureus* cells pre-exposed to teicoplanin (**Figure 41 (II)**); and 13:0 *iso* (from 0 to 9.05%), 14:0 *iso* (from 0 to 2.90%), 15:0 *iso* (from 0 to 27.38%), 16:0 *iso* (from 0 to 9.38%) and 17:0 *iso* (from 0 to 16.49%) in the case of *R. erythropolis* pre-exposed to vancomycin (**Figure 42 (II)**) and 15:0 *iso* (from 0 to 6.49%), 17:0 *anteiso* (from 0 to 19.36%) in the case of *R. erythropolis* pre-exposed to teicoplanin (**Figure 43 (II)**).

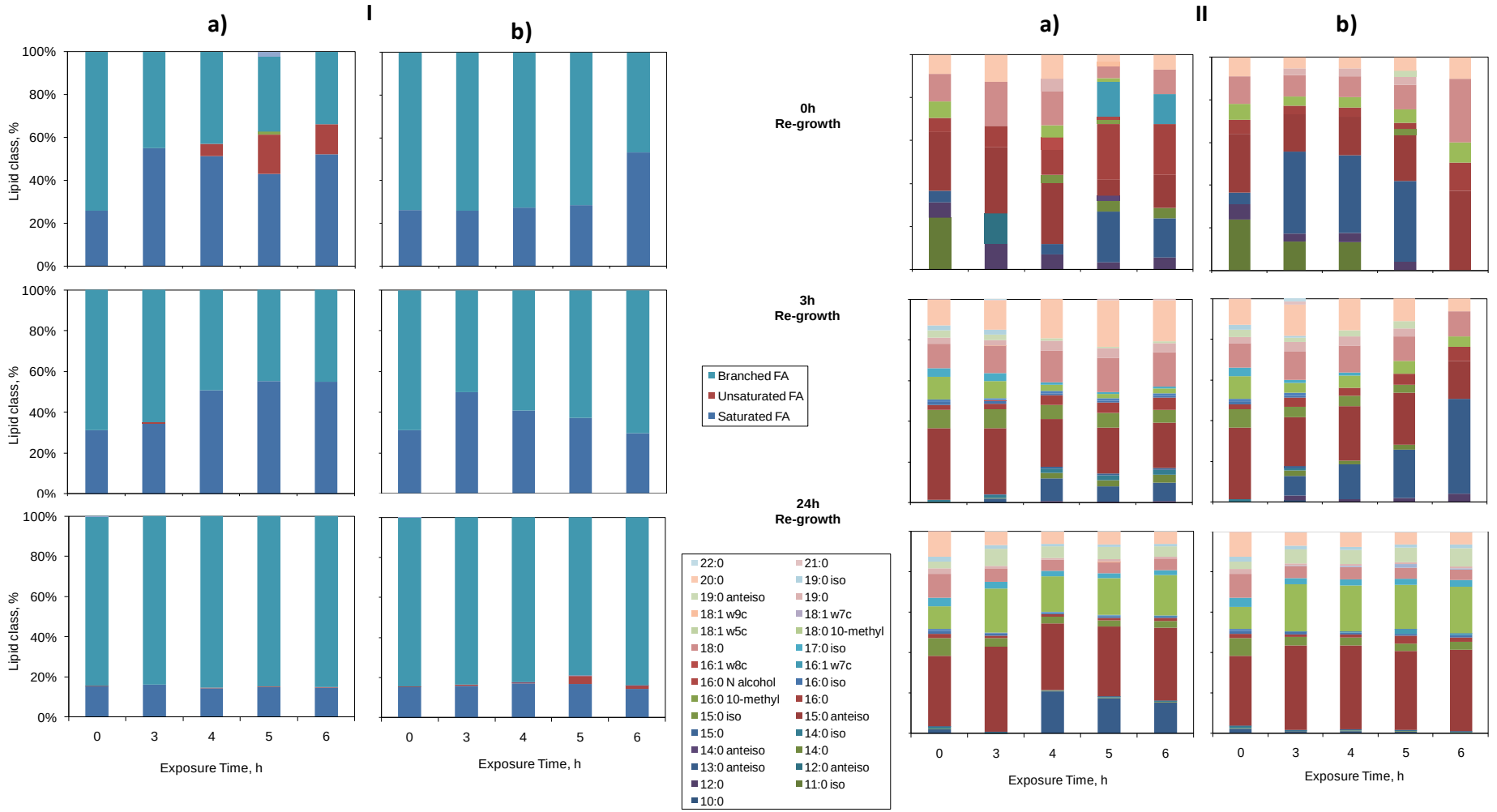

Figure 40 (I) and (II) – Lipid composition of *S. aureus* cells grown in fresh medium without antibiotic for 24 h. The cultures were inoculated with cells exposed for 6 h to 50 **(a)** and 100 $\mu g.mL^{-1}$ **(b)** vancomycin. **I:** lipid composition in terms of the chemical structure of the FA; **II:** composition in FA (only those present at concentrations higher than 1% are shown). The 'Blank' bars represent non-exposed cultures.

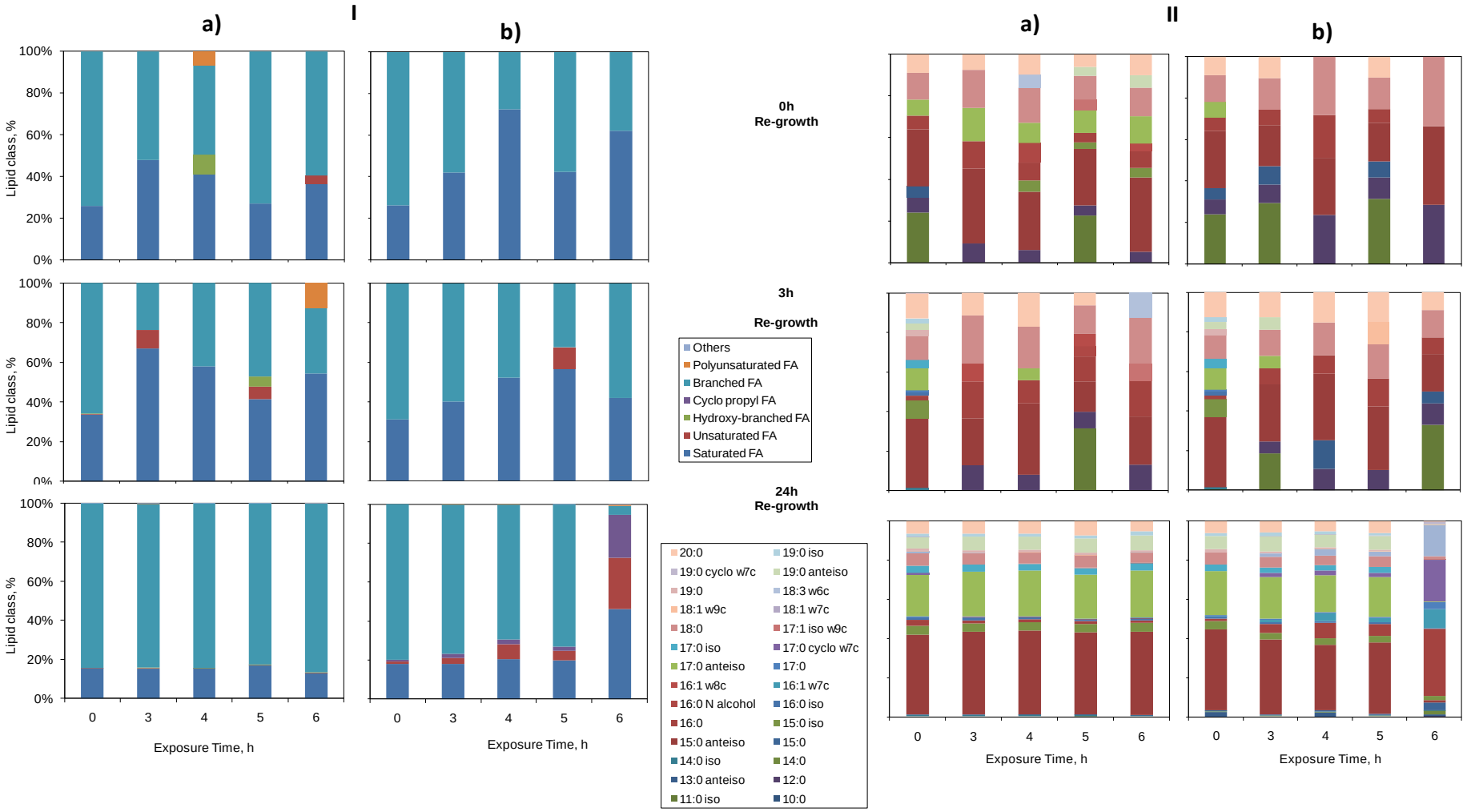

Figure 41 (I) and (II) – Lipid composition of *S. aureus* cells grown in fresh medium without antibiotic for 24 h. The cultures were inoculated with cells exposed for 6 h to 50 **(a)** and 100 µg.mL^{-1} **(b)** teicoplanin. **I:** lipid composition in terms of the chemical structure of the FA; **II:** composition in FA (only those present at concentrations higher than 1% are shown). The 'Blank' bars represent non-exposed cultures.

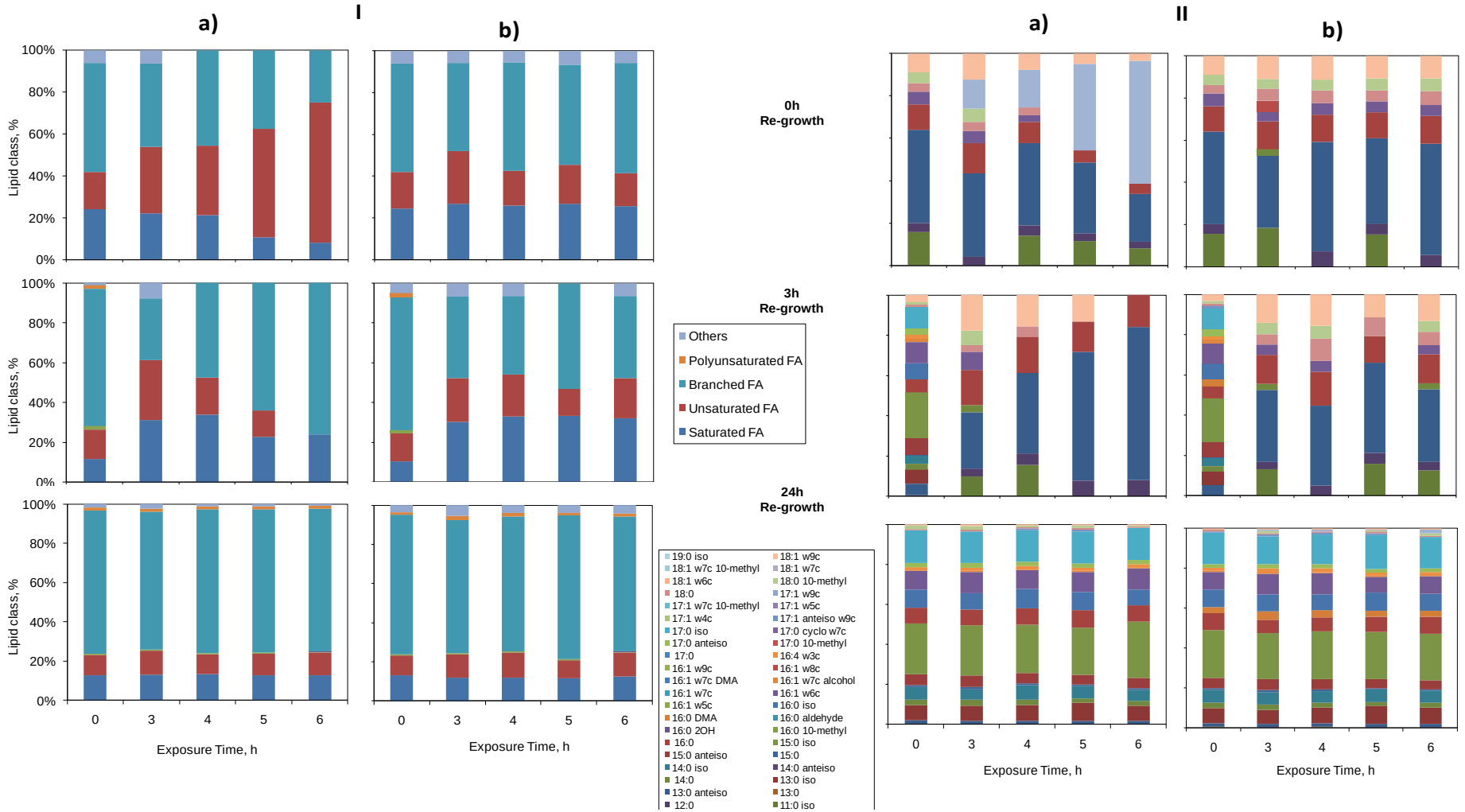

Figure 42 (I) and (II) – Lipid composition of *R. erythropolis* cells grown in fresh medium without antibiotic for 24 h. The cultures were inoculated with cells exposed for 6 h to 50 **(a)** and 100 μg.mL^{-1} **(b)** vancomycin. **I:** lipid composition in terms of the chemical structure of the FA; **II:** composition in FA (only those present at concentrations higher than 1% are shown). The 'Blank' bars represent non-exposed cultures.

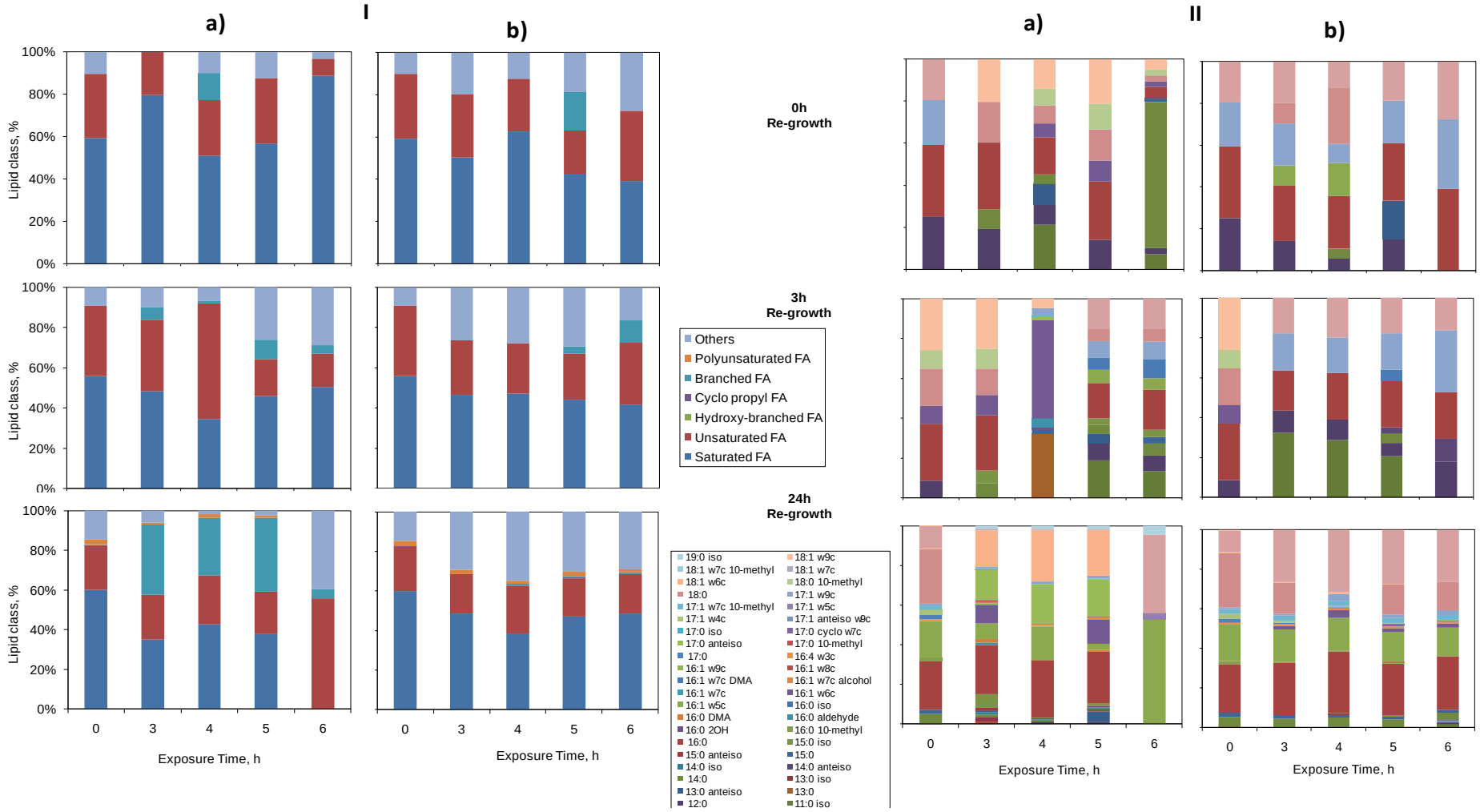

Figure 43 (I) and (II) – Lipid composition of *R. erythropolis* cells grown in fresh medium without antibiotic for 24 h. The cultures were inoculated with cells exposed for 6 h to 50 **(a)** and 100 µg.mL^{-1} **(b)** teicoplanin. **I:** lipid composition in terms of the chemical structure of the FA; **II:** composition in FA (only those present at concentrations higher than 1% are shown). The 'Blank' bars represent non-exposed cultures.

4.3.4. Zeta Potential Analysis

As for the FA composition of the cells, the changes observed at the net surface charge (**Figure 44 (I)** and **(II)**) reveal an approximation, along time, to the prior to exposure values (**Figure 12** and **Figure 13**). High antibiotic concentration and/or long periods of exposure resulted in longer times for the cells to return to their initial ZP value during the re-growth. Nevertheless, after 24 h of re-growth cells generally acquired a ZP value (-22.8 and -24.4 mV for cells pre-exposed to 50 and 100 µg.mL^{-1} vancomycin, -21.0 and -20.2 mV for cells pre-exposed to 50 and 100 µg.mL^{-1} teicoplanin in the case of *S. aureus*; -25.8 and -25.8 mV for cells pre-exposed to 50 and 100 µg.mL^{-1} vancomycin, -30 and -29.7 mV for cells pre-exposed to 50 and 100 µg.mL^{-1} teicoplanin in the case of *R. erythropolis*) similar to that of the initial, non-exposed populations (-20.0 and -29.5 mV, respectively for *S. aureus* and *R. erythropolis*).

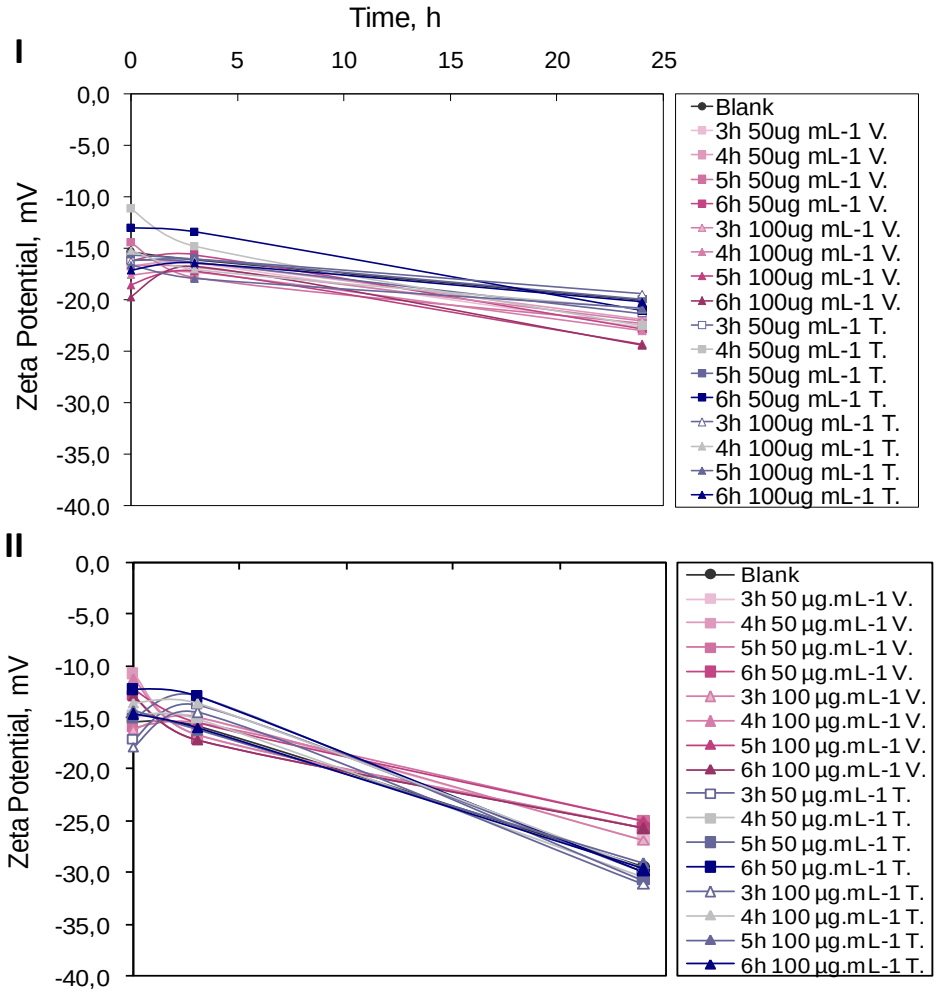

Figure 44 (I) and (II) – Zeta potential values of *S. aureus* **(I)** and *R. erythropolis* **(II)** grown in drug-free medium inoculated with the cells exposed up to 6 h to 50 (squares) and 100 µg mL^{-1} (triangles) vancomycin ('V.') or teicoplanin ('T.'). The 'Blank' curves (circles) represent non-exposed cultures.

4.3.5. Oxygen Consumption Analysis

To assess the influence of both time of exposure and concentration of antibiotics on the metabolic activity of both *S. aureus* and *R. erythropolis* persisters, the cells were previously exposed to either low or high antibiotic concentrations, for different periods of time. For that, the oxygen consumption by the cells descending from the cells which survived exposure to both low (**Figure 45**) and high (**Figure 46** and **Figure 47**) antibiotic concentrations were measured and compared to those of the non-stressed cells.

For *S. aureus* cultures pre-exposed to low antibiotic concentrations and grown in fresh, drug-free medium, similarly to what happened in the presence of antibiotics (**Figure 29**), three distinct behaviors can be observed (**Figure 45**). For concentrations lower than 12.5 $\mu g.mL^{-1}$, cells consumed high levels of oxygen (80-100%) for the first 15 h. After that period, cells likely entered the stationary phase, consuming successively less oxygen, until it ceased around 20-25 h of growth. A similar pattern was followed by the non-exposed, 'Blank' cultures, suggesting again that cells can easily tolerate these low antibiotic concentrations. A concentration of 12.5 $\mu g.mL^{-1}$ turned out to represent a transition concentration, revealing a behavior somehow between that observed for lower and higher concentrations; in the case of vancomycin exposure, the same was registered for a concentration of 18 $\mu g.mL^{-1}$. After 20-25 h of growth, cultures pre-exposed to 25 and 37.5 $\mu g.mL^{-1}$ vancomycin and 18 $\mu g.mL^{-1}$ teicoplanin started to consume high levels of oxygen. This late consume likely points towards a reduced number of initial cells, due to the bactericidal action of the antibiotics tested, and thus a long *lag* phase was required. Furthermore, after pre-exposure to 25 $\mu g.mL^{-1}$ vancomycin, the cells were able to restore their metabolic activity faster than their counterparts pre-exposed to a higher concentration of 37.5 $\mu g.mL^{-1}$, stressing the direct relation between the antibiotic concentration and the bacterial metabolic activity (*i.e.* population re-growth). The results suggest that these *S. aureus* and *R. erythropolis* cells were able to restore the initial, prior to exposure metabolic activity after 15 h of growth in fresh, drug-free medium. Moreover, for the populations pre-exposed to concentrations higher than 37.5 $\mu g.mL^{-1}$ vancomycin and higher than 18 $\mu g.mL^{-1}$ teicoplanin, oxygen consumption was never observed during the time of the experiments, supporting the results previously obtained at FA composition (**Figure 38** and **Figure 39**), and thus suggesting a higher bactericidal potency for teicoplanin.

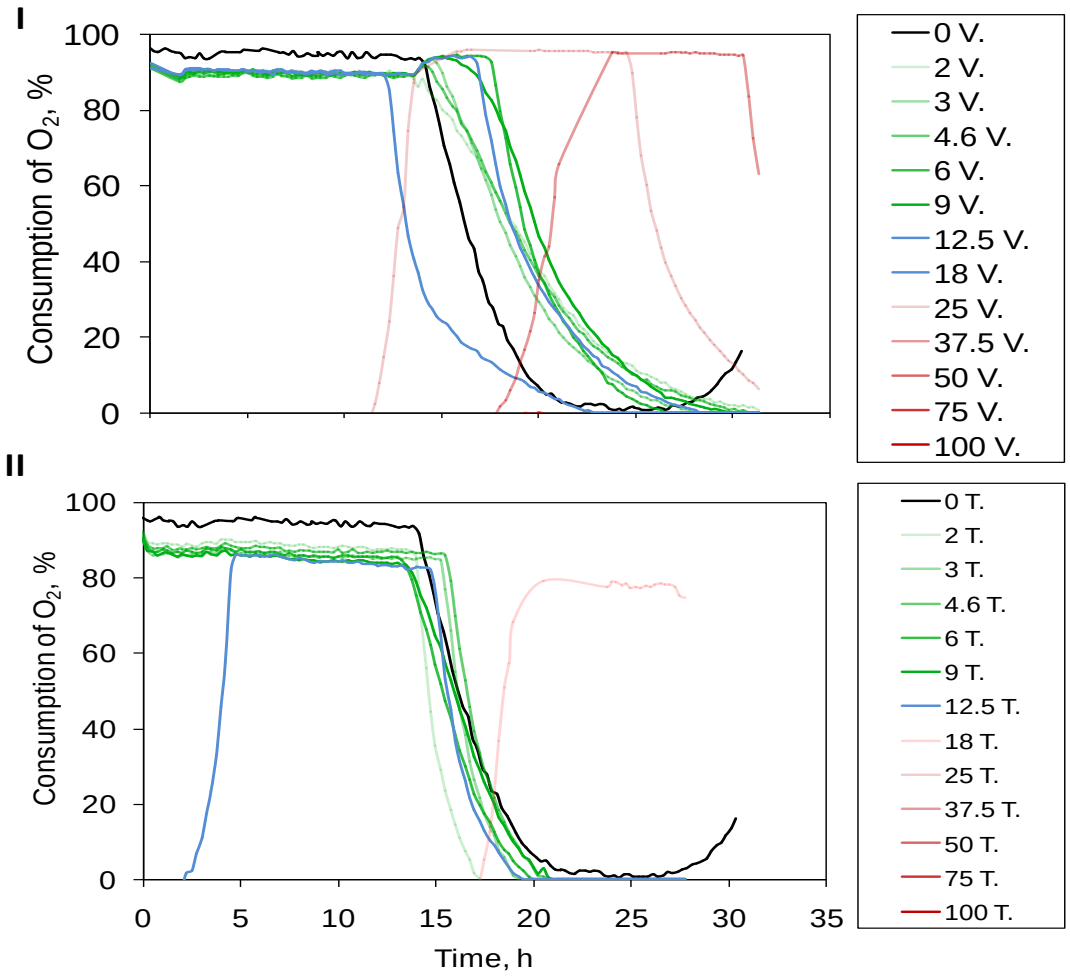

Figure 45 (I) and (II) – Oxygen consumption of *S. aureus* grown in drug-free medium inoculated with the cells exposed up to 6 h to low concentrations of vancomycin ('V.') or teicoplanin ('T.'). The 'Blank' curves represent non-exposed cultures.

For *S. aureus* cultures pre-exposed to high antibiotic concentrations and grown in fresh, drug-free medium, the same three-phase evolution was obtained (**Figure 46**). High consumption of oxygen was observed during the first 15 h for the cultures pre-exposed to 50 $\mu g.mL^{-1}$ vancomycin whilst for the last 15 h high consumption was observed for the cells pre-exposed to 100 $\mu g.mL^{-1}$ vancomycin (**Figure 46 (I)**). In the presence of high antibiotic concentrations, cells will likely increase their metabolic activity in order to develop an effective response. The results obtained during *S. aureus* growth in fresh, drug-free medium follow what was expected: when increasing the antibiotic concentration, cells will increase the oxygen consumed (in this case cells increased the time period during which they consumed high amounts of oxygen rather than the percentage of oxygen consumption), in an attempt to surpass stress action. Furthermore, the longer the time of pre-exposure, the faster cells increased their metabolic activity, suggesting the presence of regulated and sensitive response mechanism(s) against antibiotics action. In the case of pre-exposure to teicoplanin, a similar behavior was obtained in response to the increasing antibiotic concentration and time of exposure (**Figure 46 (II)**). However,

the two distinct phases – a first one for cells pre-exposed to 50 µg.mL^{-1} teicoplanin and a second one for cells pre-exposed to 100 µg.mL^{-1} teicoplanin – were not observed. Probably due to the more drastic mechanism of action of teicoplanin, which require a more radical response, the cells experienced a faster increase in the metabolic activity.

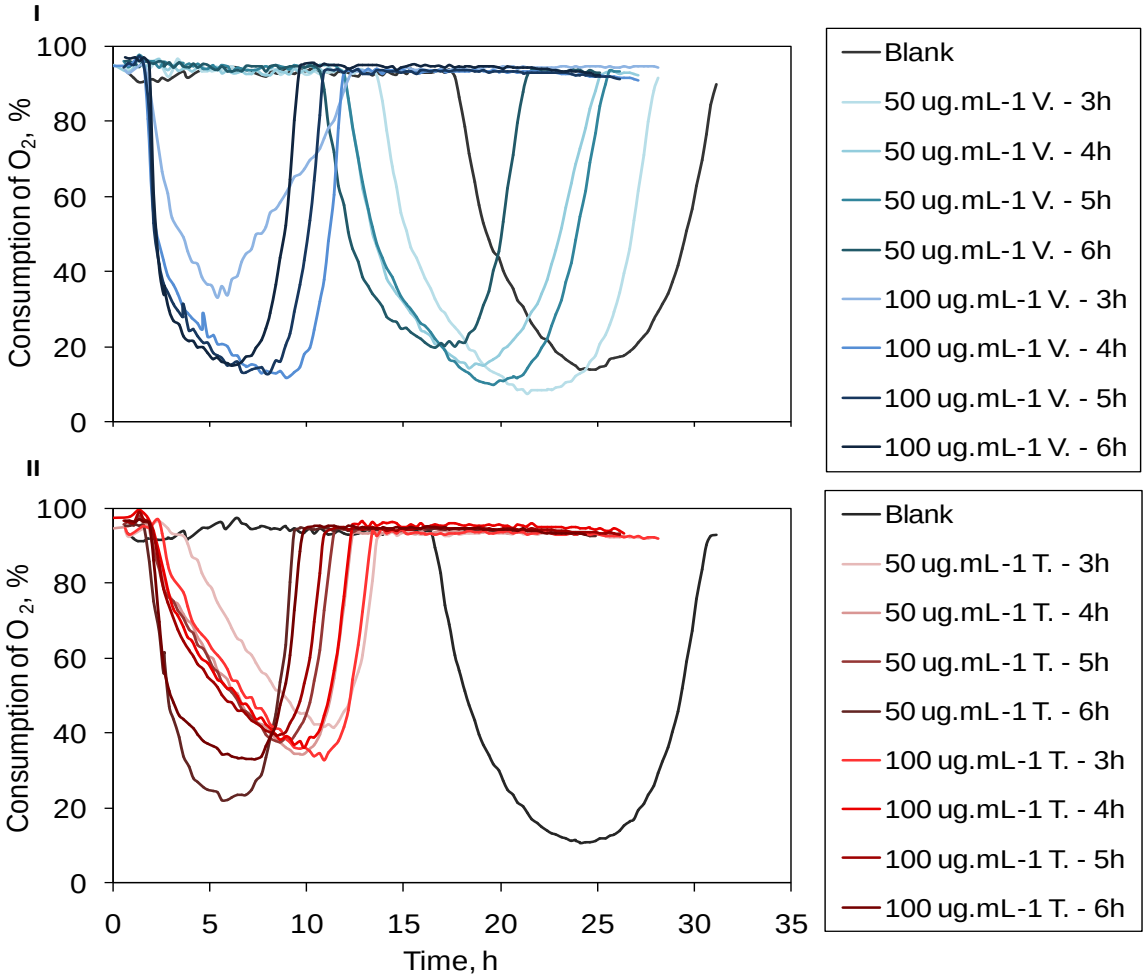

Figure 46 (I) and (II) – Oxygen consumption of *S. aureus* grown in drug-free medium inoculated with the cells exposed up to 6 h to high concentrations of vancomycin ('V.') or teicoplanin ('T.'). The 'Blank' curves represent non-exposed cultures.

The most effective response mechanisms were obtained for *R. erythropolis* (**Figure 47**). For this bacterium, and regardless of the antibiotic/concentration tested, very high levels of oxygen consumption were registered (80-100%). Following what was previously observed in the presence of antibiotics (**Figure 31**), *R. erythropolis* cells managed to maintain the metabolic activity (*i.e.* high oxygen consumption) during the re-growth, again corroborating the recent results of Orman and Brynildsen (Orman and Brynildsen, 2013). Non- or slow-growing cells, such as persister cells, can actually be metabolically active.

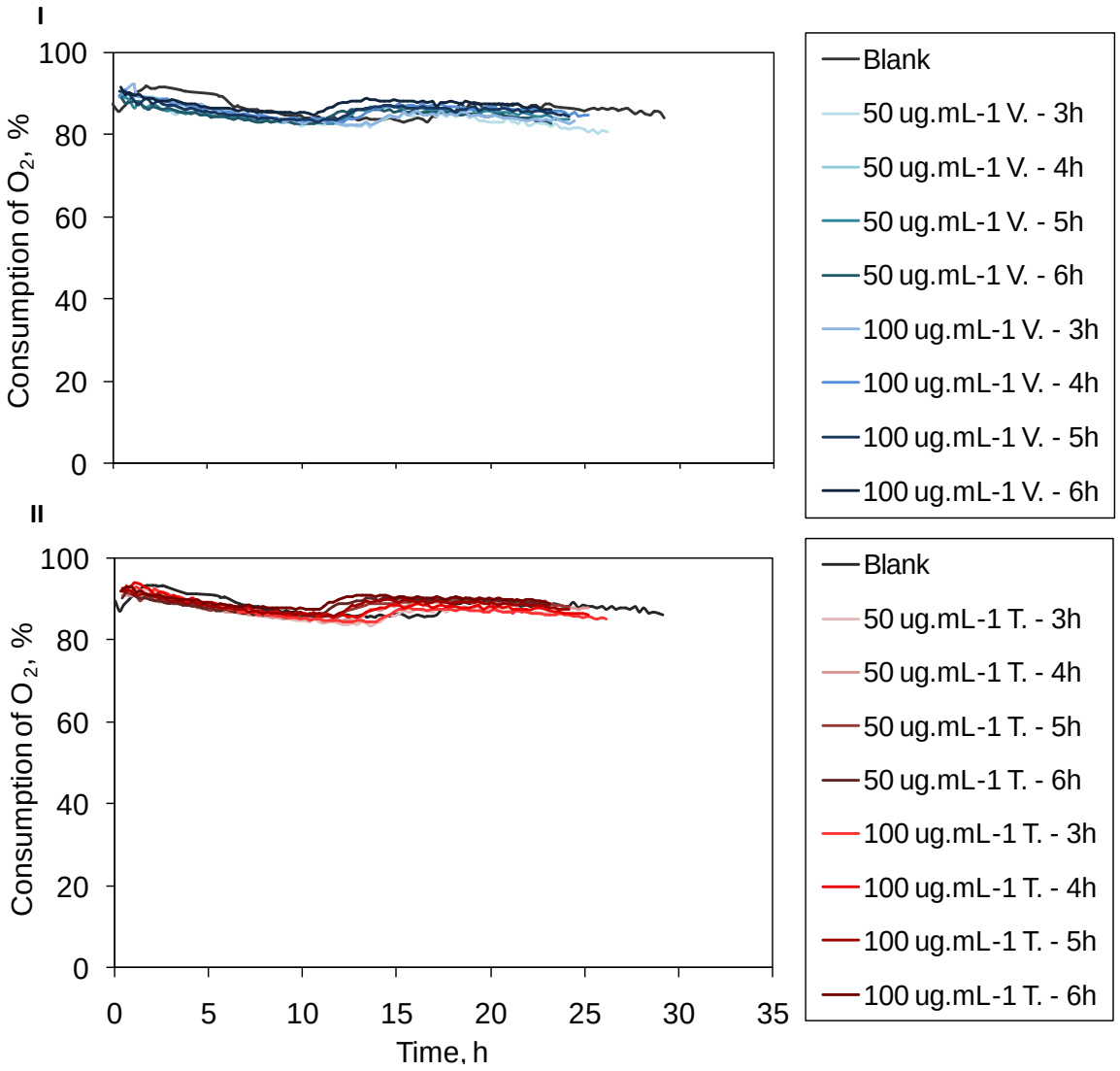

Figure 47 (I) and (II) – Oxygen consumption of *R. erythropolis* grown in drug-free medium inoculated with the cells exposed up to 6 h to high concentrations of vancomycin ('V.') or teicoplanin ('T.'). The 'Blank' curves represent non-exposed cultures.

4.3.6. Enzymatic Activity Analysis

The profile of *S. aureus* enzymatic activities during re-growth (after previous antibiotic exposure) revealed what seemed to be, at first, an unexpected enzymatic profile (**Figure 48**).

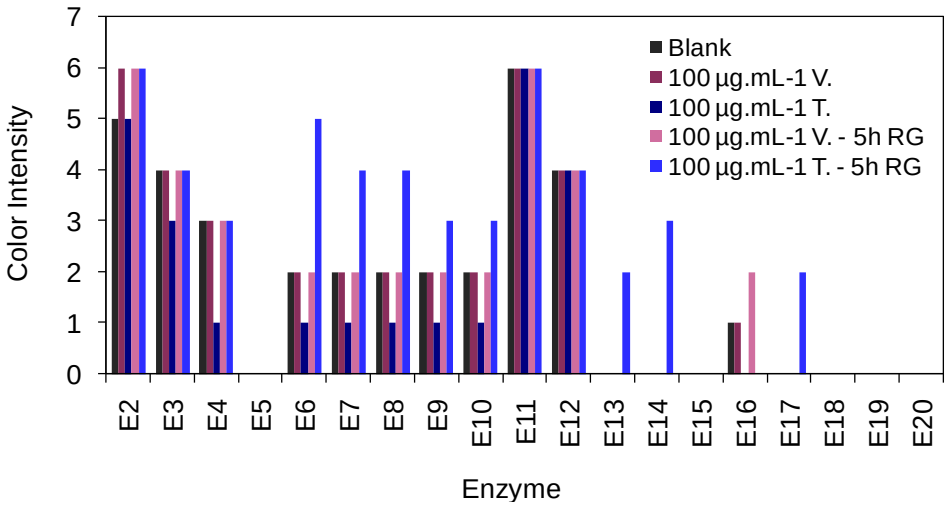

Figure 48 – Enzymatic activity of 14 enzymes in *S. aureus* re-grown for 5 h ('5h RG') in fresh, drug-free medium after 6 h of exposure to 100 µg.mL^{-1} vancomycin ('V.') or teicoplanin ('T.'). The 'Blank' bars represent non-exposed cultures. Color intensity: 0 (no activity) to 6 (strong activity). The horizontal axis corresponds to the following enzymes: E2, alkaline phosphatase; E3, esterase (C4)**;** E4, esterase lipase (C8)**;** E5, lipase (C14); E6, leucine arylamidase; E7, valine arylamidase; E8, cystine arylamidase; E9, trypsin; E10, α-chymotrypsin; E11, acid phosphatase; E12, Naphtol-AS-BI-phosphohydrolase; E1**3,** α-galactosidase; E14, β-galactosidase: E15, β-glucuronidase; E16, α-glucosidase; E17, β-glucosidase; E1**8,** N-acetyl-β-glucosaminidase; E19, α-mannosidase; E20, α-fucosidase.

For both antibiotics, 5 h of re-growth in fresh medium were sufficient for the survivor cells not only to restore the enzymatic activities previously reduced during the exposure but also to overcome, in some cases the initial, prior to exposure activity. This is particularly visible for cells surviving teicoplanin exposure. A detailed analysis shows that this increase was more pronounced for the enzymes defined as 'E2', 'E6' to 'E10', 'E13', 'E14', 'E16' and 'E17', respectively alkaline phosphatase; leucine, valine and cystine arylamidase; trypsin; α-chymotrypsin; α- and β-galactosidase; and α- and β-glucosidase. Interestingly, all of these enzymes represent hydrolases that lead, as a result of its activity, to the production and subsequently release of energy in the cell (Cannon and Hawn, 1963; Creaser, 1955; Espejo et al., 1981). Moreover, the enzyme alkaline phosphatase, which is responsible for dephosphorylation reactions (*i.e.*, removal of phosphate groups) has been related (with hyaluronidase and coagulase), to a factor of pathogenicity in *S. aureus* (Cannon and Hawn, 1963). Likewise, the activity of trypsin enzymes, also energy-producers enzymes, has also been implicated in the enhancement of *S. aureus* virulence as a direct effect of the presence of trypsin-cleavage products (Espejo et al., 1981). Curiously, the highest increases in activity were registered for the three arylamidase enzymes. Leucine, valine and cystine arylamidase catalyze the release of an N-terminal leucine, valine or cystine, respectively and as the name implies, from a wide range of peptides, amides and arylamides, producing acetyl-coA, a known 'high energy' compound essential for the

processing of many energy producing reactions in the cell. Additionally, α- and β-galactosidase enzymes proved to be bacterial adaptive enzymes, activated only in stress conditions (Creaser, 1955). Glucosidase enzymes are now known to degrade the glucose present in the MH broth starch, eventually leading to the release of energy in the cell. Moreover, β-glucosidase has been reported for some bacteria as one of the most thermostable enzymes identified up to now, often highly resistant to chemical denaturation and to degradation by proteases (Voorhorst et al., 1995). Ultimately, the observed increase in the activity of these enzymes will increase the levels of energy in the cell. These results therefore clarify the observations made during antibiotic exposure (vide section **4.2.8. Enzymatic Activity Analysis**) as strictly temporary, proving wrong the theories defending persistence as a 'dormant' phenotype. Together with the evidences collected from the oxygen consumption analysis at high antibiotic concentrations (**Figure 29**) for both *S. aureus* and *R. erythropolis*, these last data on *S. aureus* increased activities for several 'energizing' enzymes after antibiotics exposure strongly suggest an actual active state, adapted to successfully respond to the presence of stressful conditions (in this case, presence of antibiotics).

4.3.7. Overall Modifications During and After Antibiotic Exposure (Re-Growth)

Next, and to conclude, a summary of the overall modifications that cells suffered during and after antibiotic exposure, including growth in fresh medium, is given (**Figure 49** to **Figure 52**). It should be noted that the results obtained for 3 h of exposure were here selected as an example. Interestingly, and both at the level of FA composition and ZP values, the overall results clearly indicate that the survivor cells progress towards the original composition/profile, as evidenced by the great similarities between the initial levels and those obtained along the re-growth in fresh, antibiotic-free medium. Furthermore, the harsher the conditions previously experienced, the longer the period of time survivor cells will take to achieve those initial, prior to exposure levels (recall also the higher bactericidal potency previously suggested for teicoplanin relatively to vancomycin).

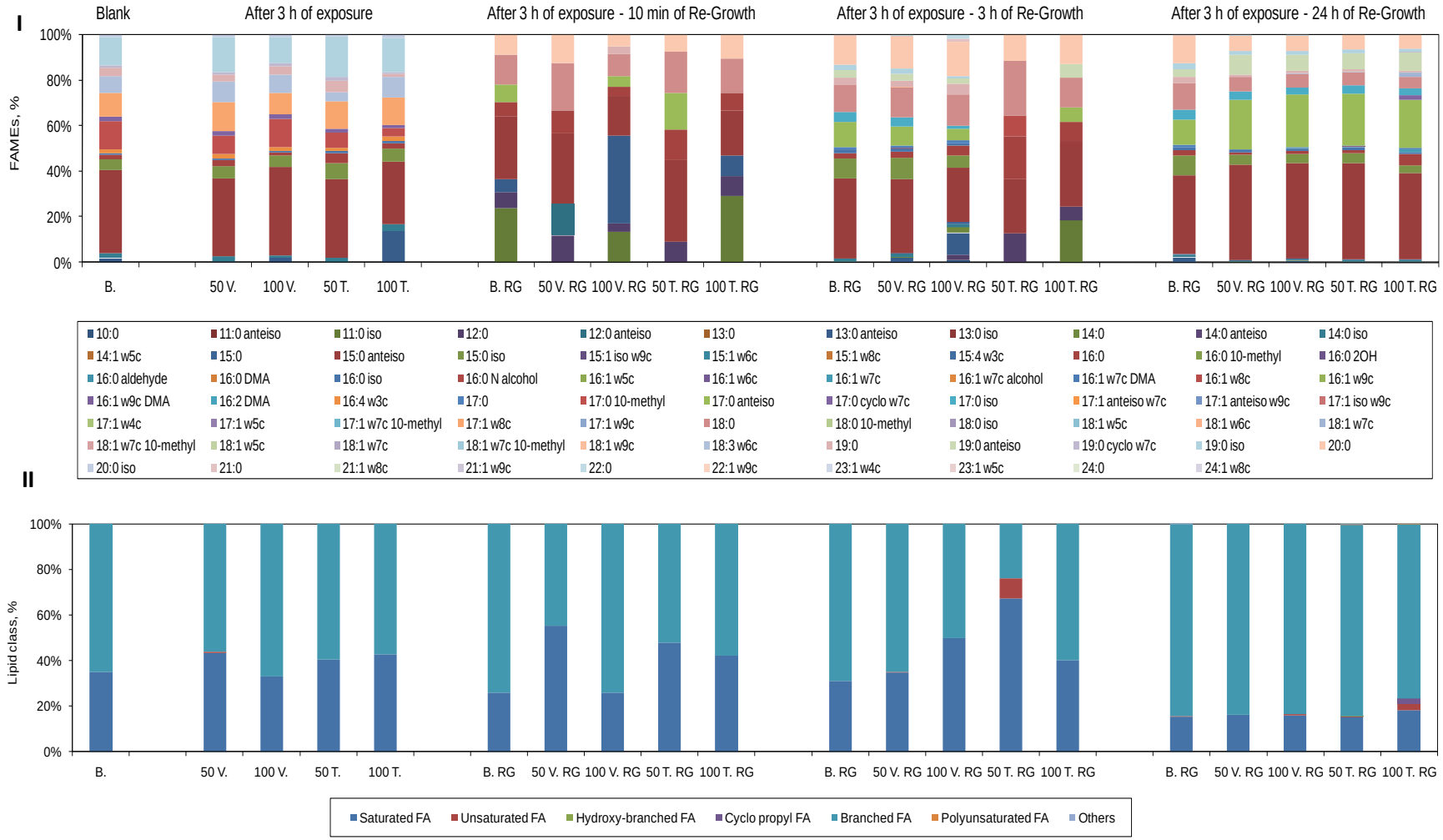

Figure 49 (I) and (II) – Summary of the effect of 3 h of exposure to high concentrations of vancomycin ('V.') and teicoplanin ('T.') on **(I)** membrane FA composition and **(II)** according to the FA molecular structure of *S. aureus* cultures The evolution of *S. aureus* FA composition after 10 minutes, 3 h and 24 h of growth in fresh, drug-free medium ('RG') is also presented. The 'B.' bars represent non-exposed cultures.

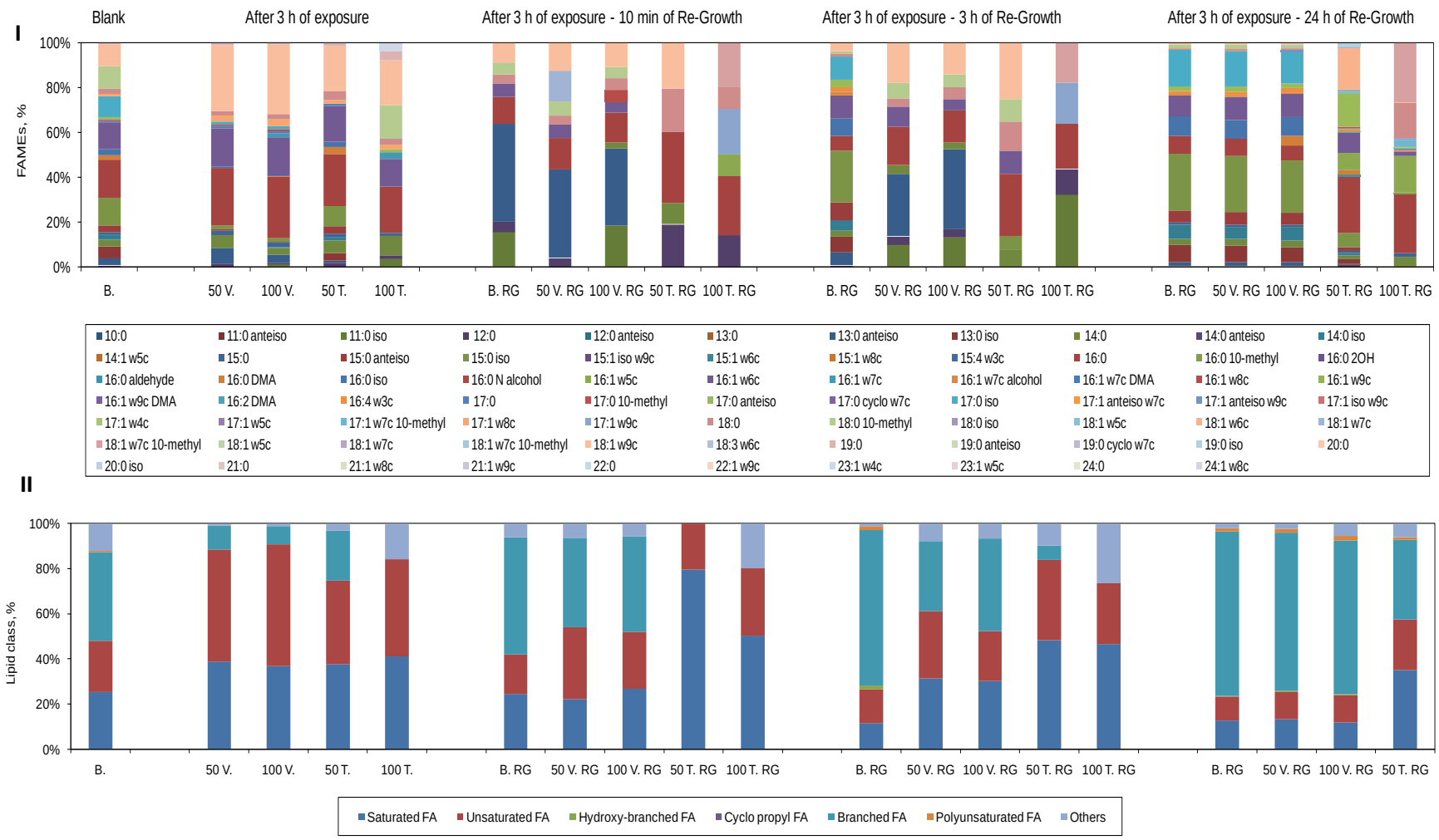

Figure 50 (I) and (II) – Summary of the effect of 3 h of exposure to high concentrations of vancomycin ('V.') and teicoplanin ('T.') on **(I)** membrane FA composition and **(II)** according to the FA molecular structure of *R. erythropolis* cultures. The evolution of *R. erythropolis* FA composition after 10 minutes, 3 h and 24 h of growth in fresh, drug-free medium ('RG') is also presented. The 'B.' bars represent non-exposed cultures.

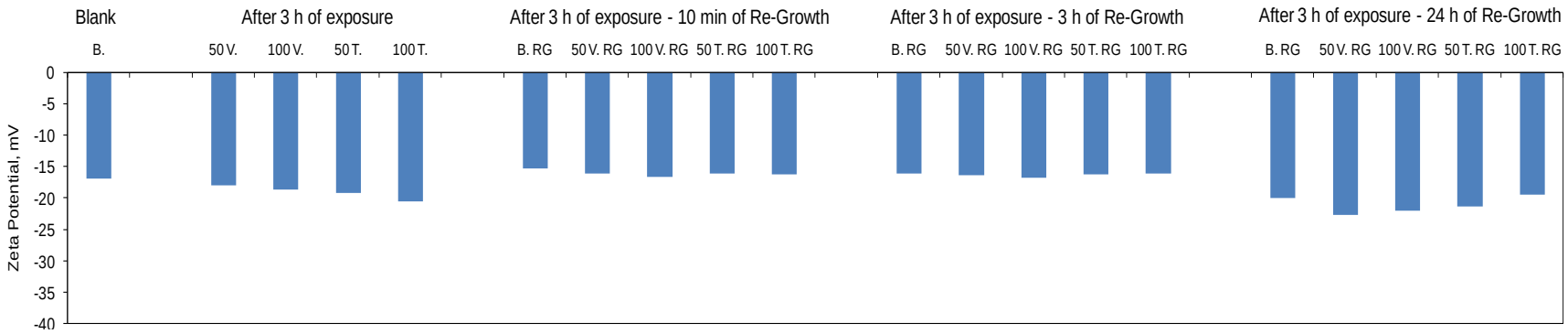

Figure 51 – Summary of the effect of 3 h of exposure to high concentrations of vancomycin ('V.') and teicoplanin ('T.') on the net surface charge of *S. aureus* cultures. The evolution of *S. aureus* ZP profile after 10 minutes, 3 h and 24 h of growth in fresh, drug-free medium ('RG') is also presented. The 'B.' bars represent non-exposed cultures.

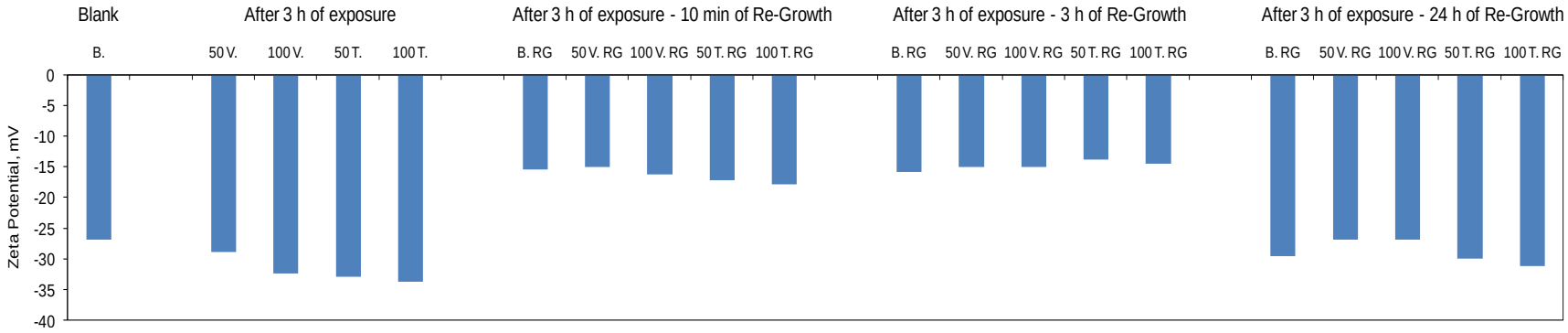

Figure 52 – Summary of the effect of 3 h of exposure to high concentrations of vancomycin ('V.') and teicoplanin ('T.') on the net surface charge of *R. erythropolis* cultures. The evolution of *S. aureus* ZP profile after 10 minutes, 3 h and 24 h of growth in fresh, drug-free medium ('RG') is also presented. The 'B.' bars represent non-exposed cultures.

5. CONCLUSION

S. aureus and *R. erythropolis* cultures were successfully grown in MHB, reaching the corresponding exponential phase in less than 3 h, with duplication times of ca. 0.71 and 0.85 h^{-1}, respectively. To promote persister formation, the cells were exposed to concentrations of both vancomycin and teicoplanin much higher than the MIC. The biphasic killing kinetics observed in both bacteria upon exposure to the two tested antibiotics/concentrations suggest that the majority of the susceptible, regular cells were killed and indicate the survival of a small fraction of tolerant, persister cells in the antibiotic exposed populations. Moreover, and despite the observation of this biphasic profile, typical for persister cells enrichment, the distinct survival rates obtained for different antibiotics/concentrations further indicates physiological heterogeneity in terms of robustness or fitness within persister subpopulations. Importantly, these different persister phenotypes (or levels of induced persistence) support that the small fraction of cells that survive antibiotic exposure is highly dependent on the specific antibiotic that is used, as recently hypothesized by others.

Relatively to the effect of antibiotics on the FA composition of the cells, both strains responded to antibiotic stress by increasing the degree of saturation of the FA of the membrane phospholipids. Contrarily, non-stressed cells increased their levels of branched FA while decreasing the percentage of saturated FA with the age of the culture. The exposed cells thereby responded to the presence of antibiotics at the lipid level as bacterial cells usual respond to a number of toxic organic compounds: by decreasing the fluidity of the cellular membrane. In the case of the net surface charge, values decreased with both time of exposure and antibiotics concentration. The more negative character of the cell surface could contribute to a repulsion mechanism towards teicoplanin but should have the opposite effect towards vancomycin. Nevertheless, an increased positive cell-wall charge as response to glycopeptides has only been observed for immediate and resistant variants of *S. aureus* (Cafiso et al., 2012).

Since the most significant alterations in FA composition and ZP values occurred during the first 3 h of antibiotic exposure, the original population should contain persister cells even before stress exposure rather as has been suggested by population dynamics studies. Notably, throughout the entire set of experiments a higher tolerance was obtained for *R. erythropolis* when compared to *S. aureus*. The higher tolerance that this bacterium has evolved towards a more effective adaptability to stress conditions, developing enhanced mechanisms of response (as shown by a large number of published studies). This could result from a higher enrichment in persister cells prior to antibiotic exposure relatively to that of *S. aureus* (as suggested by the higher survival rate during antibiotic exposure). Following these observations, the results obtained during the growth in drug-free medium of the cells which had survived exposure to high concentrations of antibiotics confirm persister cells as reversible phenotypic variants of regular cells which allow bacteria to survive a temporary environmental stress.

Finally, the levels of oxygen consumption observed upon antibiotic exposure, and the enhanced activity of enzymes related to energy production, suggest an active character of persister cells instead of the usually suggested ‘dormant’ state. Persister cells apparently promote the most effective strategy

of stress response, refuting previous hypotheses that their survival is based on ‘static’ response, deriving only from their nature.

Further work should involve: the study of bacterial response after exposure to other classes of antibiotics; and the analysis of the effect of antibiotic exposure on the action of efflux pumps and the influence of those efflux pumps in persister cells mechanism(s) of formation.

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APPENDIX

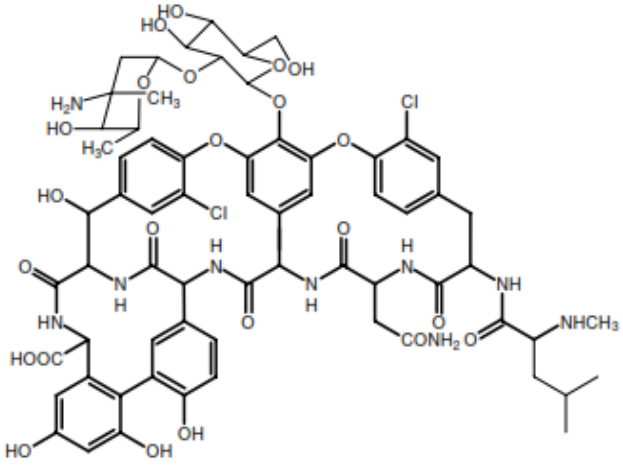

Figure A. 1 – Chemical structure of the glycopeptide vancomycin.

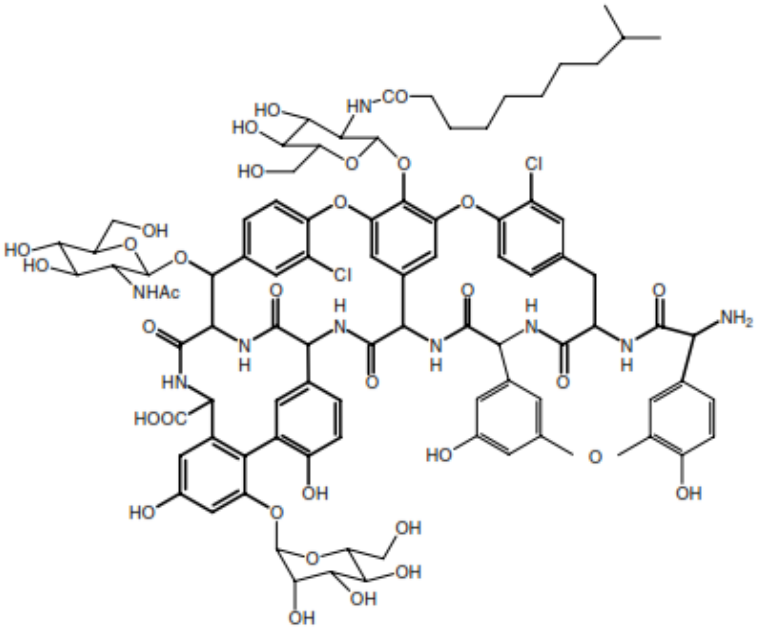

Figure A. 2 – Chemical structure of the glycopeptide teicoplanin.

Table A. 1 – Optical density data used for determination of the MIC of vancomycin in *S. aureus*.

[Vancomycin], $\mu g.mL^{-1}$	OD_{600nm}			
	16 h	**19 h**	**21 h**	**24 h**
100.0	0.181	0.111	0.105	0.128
75.00	0.211	0.091	0.096	0.091
50.00	0.184	0.121	0.124	0.115
37.50	0.174	0.099	0.100	0.090
25.00	0.178	0.121	0.120	0.140
18.75	0.182	0.111	0.104	0.100
12.50	0.181	0.112	0.117	0.126
9.375	0.181	0.112	0.110	0.103
6.250	0.177	0.116	0.105	0.101
4.688	0.182	0.107	0.105	0.104
3.125	0.207	0.316	0.516	0.667
2.344	0.199	0.482	1.202	1.249
1.562	0.183	1.089	0.782	0.690
1.172	0.179	1.106	0.846	0.778
0.781	0.179	0.753	0.408	0.535
0.586	0.177	0.399	0.522	0.484
0.391	0.291	0.507	0.452	0.583
0.293	0.281	0.424	0.354	0.441
0.195	0.531	0.718	0.422	0.420
0.146	0.275	0.468	0.470	0.458
0.098	0.372	0.476	0.499	0.486
0.073	0.372	0.394	0.396	0.448
0.049	0.364	0.496	0.583	0.550
0.037	0.378	0.432	0.904	0.548

Table A. 2 – Optical density data used for determination of the MIC of teicoplanin in *S. aureus*.

[Teicoplanin], $\mu g.mL^{-1}$	OD_{600nm}			
	16 h	**19 h**	**21 h**	**24 h**
100.0	0.176	0.135	0.138	0.123
75.00	0.181	0.095	0.099	0.097
50.00	0.179	0.295	0.332	0.387
37.50	0.186	0.133	0.124	0.115
25.00	0.183	0.131	0.149	0.141
18.75	0.182	0.119	0.123	0.143
12.50	0.187	0.132	0.129	0.117
9.375	0.184	0.125	0.137	0.128
6.250	0.185	0.121	0.113	0.107
4.688	0.181	0.140	0.138	0.117
3.125	0.181	0.104	0.102	0.098
2.344	0.189	0.125	0.119	0.111
1.562	0.187	0.098	0.097	0.101
1.172	0.182	0.348	0.261	0.304
0.781	0.184	0.173	0.202	0.269
0.586	0.180	0.335	0.400	0.460
0.391	0.185	0.279	0.263	0.293
0.293	0.280	0.437	0.408	0.512
0.195	0.287	0.233	0.615	0.374
0.146	0.279	0.476	0.468	0.625
0.098	0.287	0.375	0.375	0.370
0.073	0.381	0.460	0.495	0.484
0.049	0.382	0.482	0.519	0.583
0.037	0.323	0.604	0.541	0.721

Table A. 3 – Optical density data used for determination of the MIC of vancomycin in *R. erythropolis*.

[Vancomycin], $\mu g.mL^{-1}$	OD_{600nm}					
	16 h	**18 h**	**20 h**	**22 h**	**24 h**	**40 h**
100.0	0.136	0.149	0.180	0.172	0.161	0.122
75.00	0.104	0.104	0.094	0.089	0.084	0.076
50.00	0.127	0.121	0.289	0.297	0.296	0.288
37.50	0.100	0.086	0.087	0.088	0.096	0.078
25.00	0.102	0.099	0.091	0.090	0.099	0.093
18.75	0.103	0.102	0.094	0.096	0.137	0.147
12.50	0.095	0.093	0.090	0.089	0.083	0.089
9.375	0.087	0.085	0.082	0.083	0.083	0.080
6.250	0.088	0.090	0.085	0.090	0.108	0.114
4.688	0.087	0.084	0.083	0.083	0.080	0.087
3.125	0.087	0.088	0.087	0.084	0.084	0.086
2.344	0.086	0.086	0.084	0.084	0.083	0.083
1.562	0.084	0.084	0.083	0.082	0.077	0.086
1.172	0.086	0.086	0.088	0.086	0.087	0.126
0.781	0.123	0.131	0.142	0.151	0.143	0.347
0.586	0.155	0.166	0.191	0.195	0.202	0.449
0.391	0.152	0.173	0.182	0.203	0.191	0.419
0.293	0.169	0.186	0.196	0.219	0.295	0.682
0.195	0.153	0.181	0.186	0.236	0.213	0.500
0.146	0.165	0.199	0.215	0.229	0.278	0.652
0.098	0.146	0.157	0.172	0.178	0.180	0.511
0.073	0.154	0.180	0.194	0.205	0.211	0.590
0.049	0.148	0.155	0.170	0.176	0.181	0.482
0.037	0.151	0.172	0.185	0.198	0.204	0.525

Table A. 4 – Optical density data used for determination of the MIC of teicoplanin in *R. erythropolis*.

[Teicoplanin], µg.mL^{-1}	OD $_{600nm}$					
	16 h	**18 h**	**20 h**	**22 h**	**24 h**	**40 h**
100.0	0.147	0.149	0.153	0.226	0.319	0.345
75.00	0.175	0.189	0.145	0.136	0.131	0.140
50.00	0.203	0.197	0.185	0.217	0.215	0.256
37.50	0.140	0.147	0.143	0.131	0.134	0.085
25.00	0.157	0.193	0.214	0.225	0.275	0.311
18.75	0.157	0.182	0.157	0.144	0.150	0.105
12.50	0.125	0.115	0.114	0.107	0.105	0.097
9.375	0.103	0.109	0.099	0.101	0.099	0.093
6.250	0.093	0.095	0.093	0.094	0.088	0.090
4.688	0.099	0.100	0.107	0.109	0.113	0.198
3.125	0.093	0.089	0.089	0.087	0.087	0.092
2.344	0.109	0.112	0.122	0.132	0.145	0.343
1.562	0.109	0.112	0.120	0.128	0.149	0.430
1.172	0.136	0.131	0.142	0.183	0.211	0.444
0.781	0.134	0.134	0.125	0.142	0.164	0.384
0.586	0.176	0.175	0.220	0.291	0.386	0.648
0.391	0.138	0.144	0.162	0.209	0.271	0.637
0.293	0.185	0.198	0.256	0.337	0.407	0.705
0.195	0.147	0.167	0.184	0.270	0.297	0.688
0.146	0.210	0.229	0.304	0.374	0.457	0.846
0.098	0.142	0.151	0.166	0.202	0.238	0.622
0.073	0.177	0.186	0.211	0.245	0.280	0.603
0.049	0.152	0.154	0.182	0.197	0.240	0.694
0.037	0.178	0.192	0.197	0.232	0.350	0.930