Adaptation of *Mycobacterium* to hospital disinfectants

Raquel Cristina Teixeira Maricato

*Under Supervision of Carla C. C. R. de Carvalho and Pedro Fernandes*

*iBB – Institute for Biotechnology and Bioengineering, Department of Bioengineering*

Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

**ABSTRACT** The present work aimed to assess to morphological and phenotypical alterations, triggered by *Mycobacterium vaccae*, after exposure to hospital disinfectants; the activation of efflux pumps; and test the cross-resistance between hospital disinfectants and antibiotics. The minimal inhibitory concentrations of adapted and non-adapted *M. vaccae* cells to disinfectants were determined, and susceptibility assays were performed. The cells were exposed simultaneously to the disinfectant and to other compound. An antibiotic: levofloxacin or teicoplanin; or an efflux pumps inhibitor: thioridazine or omeprazole. Cellular aggregation and adjustment of the fatty acids composition were alterations observed during the experiments. Furthermore, the adapted cells showed to be more susceptible than non-adapted cells, since adapted cells were only able to grow in presence of omeprazole. Additionally, non-adapted cells exposed to levofloxacin showed a better growth than the control cells. These results suggest that non-adapted bacteria made use of efflux pumps, specifically in the case of levofloxacin, whereas with adapted bacteria possibly occurred cross-resistance between omeprazole and disinfectants. Nevertheless, cannot be excluded the occurrence of other adaptive mechanisms.

**Keywords:** *Mycobacterium*, Hospital disinfectants, Efflux pumps, Antibiotics, FAMEs

**I. INTRODUCTION**

The main target of the antibacterial agents, organic solvents and biocides is the cell wall and cytoplasmic membrane. Therefore, it is not coincidence that one of the mechanisms of adaptation displayed by mycobacteria in response to toxic compounds is the alteration of the cell wall and membrane composition [1]–[3].

In addition, other adaptive mechanisms described for mycobacteria comprise cell aggregation, changes of the physicochemical properties through the increase of the hydrophobicity of the cell surface, production of drug-modifying and drug-inactivating enzymes, efflux systems and low permeability of the cell wall [4]–[8].

The unique mycobacteria cell wall plays an important role in pathogenicity, due to its structure which confers them low permeability the antibiotics have not any effect upon these bacteria [8], [9].

Furthermore, mycobacteria resistance to antibiotics has been growing, becoming a great concern, and the problem rise with multidrug resistant tuberculosis (MDR-TB) which is a *Mycobacterium tuberculosis* strain that is resistant to at least isoniazid and rifampicin, two first line antibiotics, and with extensively drug resistant tuberculosis (XDR-TB) which is caused by a *M. tuberculosis* strain that is also resistant to any fluoroquinolone and at least one of the three injectable drugs capreomycin, kanamycin, and amikacin [10], [11].

Under stress conditions, bacteria struggle to maintain the integrity and fluidity of the membrane [7], [12], [13]. To adjust membrane fluidity bacteria entail modifications in the fatty acids (FAs) composition through changing the saturation degree of the FAs, cis and trans isomerisation, iso- and anteiso-branching and chain length modification [13], [14]. Additionally, the adaptive response depends on the category of the chemical compound. When grown in presence of the long-chain alcohols bacteria increase their degree of saturation, since long-chain alcohols penetrate deep into the cell membrane. In opposition, short-chain alcohols can only penetrate lightly into hydrophobic phospholipid bilayer, causing an enlargement on the hydrophilic headgroups, and bacteria respond inserting unsaturated FA [15].

Nonetheless, to modify the FAs composition, bacteria need to be able to perform *de novo* synthesis, which is only possible while bacteria are growing. Otherwise, processes of biosynthesis of FAs and adjustments by saturation degree will be prevented. Some bacteria are also able to accomplish the conversation of saturated FAs into unsaturated FAs using desaturase enzymes [12], [15], [16].

Efflux pumps promote the extrusion of drugs and might be specific for a certain type of compounds or might export a broad range of compounds, which can be chemically and structurally unrelated [17]–[19]. These efflux systems, due to their different mode of action might be classified into five families: the (ATP)-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the resistance-nodulation-division (RND), the small multidrug resistance (SMR), and the multidrug and toxic compound extrusion (MATE) [20]–[23].

Mycobacteria present resistance to INH, RIF, fluoroquinolones (e.g. levofloxacin), ethambutol, β-lactams and streptomycin [3]. Several efflux pumps have been characterized to mycobacteria, in particular for *M. tuberculosis*, as antibiotic transporters [24]. However, using efflux pump inhibitors (EPIs) some of the antibiotic resistance can be reverted [25]. Respertine, verapamil, thioridazine, omeprazole, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and ortho-vanadate are some of the EPIs known [17], [26], [27].

Moreover, thioridazine, is an ABC efflux pump inhibitor, inhibiting Ca²⁺ transport prevents the functioning of the
enzymes involved in generating cellular energy [28], and omeprazole is a MFS transporter inhibitors which is H+/K+ ATPase pump inhibitors [29].

It is also known that mycobacteria have resistance to certain disinfectants and antiseptics. The commonest mycobacteria involved in hospital infections are *M. chelonea* and *M. chelonae*-like organisms which were found in dialysis, specific types of surgery and post-injection abscesses, and *M. avium-M. intracellulare* and *M. furtuitum* can infect immunocompromised patients [30]–[33].

The disinfectants and antiseptics widely used for infection control practices in hospital and health care settings for prevention of nosocomial infections include alcohols, aldehydes, iodophors (e.g. povidone-iodine), biguanides (e.g. chlorohexidine) and bisphenols [30], [32], [34]–[37].

The present work assesses to the possibility of efflux pumps extrude hospital disinfectants and provide information about the possible cross-resistance of antibiotics and hospital disinfectants, through determination of the minimal inhibitory concentration of mycobacterial cells adapted and non-adapted to disinfectants in presence of antibiotics and in presence of EPIs. In addition, fluorescence microscopy was used in order to evaluate the morphologic and phenotypic alteration during the adaptation of *M. vaccae* to hospital disinfectants.

### II. MATERIALS AND METHODS

#### a. Materials

Mueller Hinton was purchase from Sigma-Aldrich (Fluka Analytical). Mueller Hinton Broth (23g/L) was prepared in distilled water, autoclaved at 121°C for 20 minutes and stored at 4°C. Tween 80 was purchase from Merck-Shuchardt, and ethyl acetate ≥99.7% was purchased from Fluka. The antibiotics, Levofloxacin and Teicoplanin, and the Efflux Pump Inhibitors (EPIs), Thioridazin and Omeprazol were purchased from Sigma-Aldrich. Levofloxacin (1mg/mL), teicoplanin (1 mg/mL and 10mg/mL), and thioridazin (1 mg/mL and 10 mg/mL) stock were prepared in mili-Q water, whereas omeprazol stocks (4.5 mg/mL and 10 mg/mL) were prepared in absolute ethanol (>99.9%, from Panreac) and in dimethyl sulfoxide (DMSO) purchased from Merck, respectively.

#### b. Bacterial Strain and Growth Conditions

The strain studied was *Mycobacterium vaccae* ATCC15483. The strain is deposited and maintained at the iBB - Institute for Biotechnology and Bioengineering, Instituto Superior Técnico, Lisbon, Portugal. Mycobacterial cells were grown in 100 mL Erlenmeyer flasks containing 20 mL of Mueller Hinton Broth medium supplemented with 0.1% of Tween 80, at 30°C and 200 rpm on incubator Agitorb 200 (Aralab). The mycobacterial growth was monitored by optical density at 600 nm (OD600) using a Spectrophotometer U2000 (Hitachi).

#### c. Bacterial Adaptation to Disinfectants

The disinfectants used in this assay were Aniosrub and gel alcohol. Three pulses of each disinfectant, in a concentration of 2.5%, were added to 40 mL of cell culture in Mueller Hinton Broth medium. The first addition was made when cells reached the mid-exponential phase, and the subsequent additions were performed when the cells, monitored by measurement of OD600, showed to be recovering and still growing. Assays were carried out in duplicate. The adapted mycobacterial cells were collected and used in 96-well plates to determine the Minimal Inhibitory Concentrations (MICs) of antibiotics and EPIs and to assess their re-growth in Erlenmeyer flasks with ½ or ¼ of the determined MICs.

#### d. Minimal Inhibitory Concentration Determination

MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2014). In summary, antibiotics and EPIs were serially diluted in 96-well microplates (Sarstedt Inc., Newton, USA) in Mueller Hinton broth. A volume of 50 μL of exponential cell culture diluted to 0.5 McFarland was added at each well containing 150 μL of medium and antibiotic or efflux pump inhibitor. The microplates were incubated at 30°C and MICs were determined after 72h of exposure to the antibiotics and EPIs by measurement of OD600 using a SpectraMax® Plus 384 Microplate Reader spectrophotometer from Molecular Devices (Silicon Valley, Ca, USA). The solution stocks of antibiotics and EPIs used for these assays were the following: 1 mg/mL (levofloxacin, teicoplanin, and thioridazin), 4.5 mg/mL (omeprazol).

1. **MICs using Adapted Cells**

For adapted mycobacterial cells, two plates were used: one for cells adapted to Aniosrub and another for cells adapted to gel alcohol. For both cases, levofloxacin was diluted from 10 to 0.01 μg/mL, teicoplanin was diluted from 100 to 0.098 μg/mL, thioridazin was diluted from 149.3 to 0.146 μg/mL, and omeprazol was diluted from 500 to 0.488 μg/mL. The MICs determined were the following for cells adapted to aniosrub: MIC<sub>levofloxacin</sub> = 5 μg/mL, MIC<sub>teicoplanin</sub> >100 μg/mL, MIC<sub>thioridazin</sub> >149.3 μg/mL, and MIC<sub>omeprazol</sub> = 500 μg/mL; and for cells adapted to gel alcohol: MIC<sub>levofloxacin</sub> = 1.25 μg/mL, MIC<sub>teicoplanin</sub> >100 μg/mL, MIC<sub>thioridazin</sub> =149.3 μg/mL, and MIC<sub>omeprazol</sub> = 500 μg/mL.

2. **MICs using Non-adapted Cells**

For non-adapted mycobacterial cells, two plates were used: one for MIC determination with antibiotics and other for MICs of EPIs. Levofloxacin was diluted from 10 to 0.01 μg/mL and 7.5 to 0.007 μg/mL, teicoplanin was diluted from 100 to 0.098 μg/mL and 75 to 0.073 μg/mL. Thioridazin was diluted from 149.3 to 0.073 μg/mL and 125 to 0.061 μg/mL, and omeprazole was diluted from 500 to 0.244 μg/mL and...
400 to 0.195 μg/mL. The MICs determined were the following: MIC\textsubscript{levofloxacin} = 0.625 μg/mL, MIC\textsubscript{teicoplanin} >100 μg/mL, MIC\textsubscript{thioridazin} = 18.7 μg/mL, and MIC\textsubscript{omeprazole}= 250 μg/mL.

e. Susceptibility Test

i. Adapted Cells

After determination of the MICs the mycobacterial cells were grown in 20 mL of Muller Hinton Broth. For each cell culture previously adapted to disinfectants (aniosrub and gel alcohol), 5 Erlenmeyer flasks were used. To each Erlenmeyer flask, 4 mL of the adapted cell culture were added. One Erlenmeyer flask was used as control (only disinfectant), and the other 4, in addition to disinfectant, contained: ½ of the MIC of levofloxacin, ¼ of the MIC of teicoplanin, ½ of the MIC of thioridazin, and ½ of the MIC of omeprazole. The solution stocks used were the following: 1 mg/mL (levofloxacin and teicoplanin), 10 mg/mL (thioridazin and omeprazole).

ii. Non-adapted Cells

For the non-adapted cells, a similar procedure was applied. To non-adapted cell cultures, 5 Erlenmeyer flasks were used, the first for control containing only disinfectant, and the other 4 as following: ½ of the MIC of levofloxacin, ½ of the MIC of teicoplanin, ¼ of the MIC of thioridazin, and ½ of the MIC of omeprazole. The solution stocks used were the following: 1 mg/mL (levofloxacin and teicoplanin), 10 mg/mL (thioridazin and omeprazole). The growths were monitored by measurement of OD\textsubscript{600}.

f. Fatty Acids Analysis

To 1.5 mL eppendorfs (Eppendorf), 1 mL of cell suspension from each culture was collected. After centrifugation (μSpeedFuge SFA13K Microcentrifuge, Savant Technologies) at 10,000 g during 5 minutes, the supernatant was discarded, the cells were recovered and washed by vortex with 1 mL of mili-Q water and centrifuged again. The washing step was repeated twice to avoid interference of disinfectant on the analysis. The fatty acids of the cells samples were extracted and methylated to fatty acids methyl esters (FAMEs) using the Instant FAME kit from MIDI Inc. (Newark, USA). FAMEs analysis was carried out by gas chromatography using a 6890N gas chromatograph from Agilent Technologies (Palo Alto, CA, USA), with a flame ionization detector and a 7683B series injector, and equipped with a 25 m long Agilent J&W Ultra 2 capillary column from Agilent. The FAMEs were identified by MIDI Sherlock\textsuperscript{®} software version 6.2, using the method PLFAD1.

g. Fluorescence Microscopy and Image Analysis

i. Cell Viability

500μL of cell suspension was collected from the culture and the cells were stained using a LIVE/DEAD\textsuperscript{®} BacLight\textsuperscript{TM} bacterial viability kit, purchased from Molecular Probes (Invitrogen Co., Carlsbad, California, USA). From this cell suspension, a drop of 0.5μL was used to observe cell viability. The kit contains a mixture of SYTO\textsuperscript{®} green fluorescent nucleic acid stain and propidium iodine (non-viable cells are stained red and viable cells are stained green). The mycobacterial cells were observed by fluorescence microscopy using a Olympus CX40 microscope, with an Olympus U-RFL-T burner and an U-MWB mirror cube unit (excitation filter: BP450-480; barrier filter: BA515). Images were captured by an Evolution\textsuperscript{TM} MP5.1 CDC colour camera using the software Image-Pro Plus, both from Media Cybernetics, Inc. (USA).

III. RESULTS AND DISCUSSION

Bacteria are able to modify their fatty acids (FAs) composition in response to environmental stresses or when reaching the stationary phase. The alteration of FA is one of the main adaptation mechanisms applied by these organisms, by changing the structure and the amount of lipids to maintain the fluidity and integrity of the membrane [15], [38]. However, this is not the only mechanism used by bacteria, cellular aggregation and activation of efflux pumps are other examples.

In this study, M. vaccae cells were adapted to the disinfectants, gel alcohol and Aniosrub (Figure 1). The disinfectants were added to each cell culture in a concentration of 2.5% (v/v). From the FA analysis, bacterial cells was showed an tendency to adjust the membrane in order to reduce the fluidity of the membrane, in both disinfectants, due to the increase of iso-branched FA, like it was described for Rhodococcus erythropolis [39].

![Figure 1 – Adaptation growth curves of M. vaccae to gel alcohol and Aniosrub. Orange arrows indicate disinfectant addition.](image-url)

Before to proceed with the minimal inhibitory concentration (MIC) determination and susceptibility assays, M. vaccae cells were visualized by fluorescence microscopy to evaluate the morphological and phenotypical alterations and verify their viability. As can be observed in Figure 2, cellular aggregation (A, C and D) occurred in exposure to both disinfectants, which indicates that cells displayed this type of response to deal with the environmental condition imposed, and probably the cells might have changed the hydrophobicity of their cell surface causing the aggregation.
Moreover, the cells involved in the aggregate in C showed a more elongated form. This is in accordance with a recent study, which suggests that bacterial cells might elongate their form under antibiotic stress [40]. Nevertheless, it was also visualized dispersed bacterial cells with a rod-shape morphology, which it is expected for these bacteria (B).

Furthermore, bacteria might activate efflux pumps to extrude antibiotics and other toxic compounds from the interior of the cell, being the efflux pumps one of the main mechanisms responsible for antibiotic resistance [41]. In the present study to determine the susceptibility of M. vaccae, the MICs were determined for the antibiotics (levofloxacin and teicoplanin) and EPIs (thioridazine and omeprazole). The values of MICs obtained, depended on the conditions imposed to the cells, adapted cells to disinfectants or non-adapted cells, as demonstrated in Table 1. To guarantee the viability of the cells, the susceptibility assays were performed with concentrations of antibiotics and EPIs below the MIC.

<table>
<thead>
<tr>
<th>Table 1 – Values of MICs of antibiotics and EPIs determined for M. vaccae.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MIC (µg/mL)</strong></td>
</tr>
<tr>
<td><strong>Antibiotics</strong></td>
</tr>
<tr>
<td>Levofloxacin</td>
</tr>
<tr>
<td>Teicoplanin</td>
</tr>
<tr>
<td><strong>EPIs</strong></td>
</tr>
<tr>
<td>Thioridazine</td>
</tr>
<tr>
<td>Omeprazole</td>
</tr>
</tbody>
</table>

The susceptibility assays were performed with adapted cells to gel alcohol and Aniosrub in presence of ½ of MIC of levofloxacin or EPIs, and in presence of ¼ of the MIC of teicoplanin. And non-adapted cells were exposed to ½ of the MIC for all the antibiotics or EPIs used in the study.

From the re-growth of the adapted cells to gel alcohol exposed simultaneously to the disinfectant and to the respective antibiotic or EPI (Figure 3), apart to the control cells, it was only observed growth in cells exposed to omeprazole, and in regard to the thioridazine cells condition, they seemed started to grow after 50 h of the assay, but become to decline 15 h later. Regarding to the antibiotics conditions no significant grow was observed.

In opposition, non-adapted cells exposed simultaneously to gel alcohol and to antibiotic or EPI (Figure 4), it was observed growth in all the condition, with a highlight to the levofloxacin cells condition, which showed to grow even more than the control cells. The thioridazine cells condition also showed a better growth than the control cells from 15 to 40 h oh the assay.

The information given by these data indicates that non-adapted cells were more able to overcome the stressful environment imposed.

In order to understand if M. vaccae cells performed alterations at the level of the fatty acids of the membrane composition, it was collected samples for fatty acids methyl esters (FAMEs) analysis. These results are shown in Figure 5 for adapted cells to gel alcohol and exposed to antibiotic or EPI, and in Figure 6 for non-adapted cells exposed to gel alcohol and to antibiotic or EPI.

From Figure 5, it is observed that the control cells and the
Figure 5 – Lipid composition of *M. vaccae* cells previously adapted to gel alcohol grown in the presence of ½ of the MIC of the EPIs and levofloxacin, and ¼ of the MIC of teicoplanin. The fatty acids are grouped accordingly to their type: MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids), SSFA (straight-chain fatty acids), BSFA (branched-chain fatty acids), 10-methyl fatty acids, and other fatty acids. The ‘Gel Alcohol’ represents the control of the assay where the cells were only exposed to the disinfectant.

Figure 6 – Lipid composition of non-adapted *M. vaccae* cells previously adapted to gel alcohol grown in the presence of ½ of the MIC of the antibiotics and EPIs. The fatty acids are grouped accordingly to their type: MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids), SSFA (straight-chain fatty acids), BSFA (branched-chain fatty acids), 10-methyl fatty acids, and other fatty acids. The ‘Gel Alcohol’ represents the control of the assay where the cells were only exposed to the disinfectant.
levofoxacin and to the antibiotic or EPI (Figure 7), similarly to it was verified to gel alcohol, only cells exposed to omeprazole the cells have grown, although started to decline after 45 h of growth.

The growth of non-adapted cell to Aniosrub and exposed simultaneously to the antibiotic or EPI is presented in Figure 8. To exception of cells exposed to levofoxacin, it was observed growth in all the conditions.

These observations suggest that non-adapted cells deal better with the stress imposed by the addition of two toxic compounds at the same time than adapted cells.

In Figure 6 are presented the alterations in the fatty acids composition of non-adapted cells to gel alcohol exposed to the different conditions. From these data, it is showed which the trend in all causes was to decrease the MUFA. Furthermore, in the control, levofoxacin and thioridazine it was also observed an increase of BSFA, especially after a prolonged time of exposition. In the control and thioridazine cells, it was due to the contribution of the iso-branched FA, whereas in levofloxacin cells, the increase of the BSFA, it was due to the great contribution of the anteiso-branched FA, specifically the 13:0 anteiso. These suggest that in teicoplanin cells, the adjustment was made in a way to maintain the flexibility. In thioridazine cells, after 64 h, the trend it was for decrease the flexibility of the membrane, and omeprazole cells condition, in opposition to all the other conditions, functioned in a way to increase the fluidity of the cellular membrane.

Through these data, it can be concluded that non-adapted cells to gel alcohol had a better capacity to survive to the environment imposed in comparison to the adapted cells. In addition, levofloxacin belongs to the fluoroquinolones class of antibiotics. Several efflux pumps have been described for mycobacteria, and it is known the existence of efflux pumps that extrude fluoroquinolone antibiotics, the Rv2686c-2687c-2688c and Rv1634 is an efflux pump described for M. tuberculosis, and the PstB and LfrA are pumps described for M. smegmatis [17], [27], [42], [43], considering the possibility of M. vaccae also present homologous efflux pumps, this organism might have been able to extrude levofoxacin, namely the non-adapted cells. However, for this hypothesis to be true the adapted cells should have showed a similar behaviour, or other hypothesis, it is that the prolonged exposure to the disinfectant might have influenced the response to levofoxacin. Regarding to the omeprazole condition, in adapted cells, it seemed to have occurred cross-resistance, but other mechanisms should not to be excluded.

In regard to the susceptibility assay of adapted M. vaccae cells to Aniosrub exposed simultaneously to the disinfectant

Figure 7 – M. vaccae previously adapted to Aniosrub grown in the presence of ½ of MIC of levofloxacin and EPIs, and ¼ of MIC of teicoplanin. Aniosrub curve is the control.

Figure 8 – Non-adapted M. vaccae grown in the presence of ½ of MIC of antibiotics and EPIs. Aniosrub curve is the control.

The FAMEs analysis regarding to the adapted mycobacterial cells to Aniosrub is shown in Figure 9. From the analysis of the figure, it is showed that in the control cells, the amount of MUFA decreased over time, while the BSFA increased with a higher proportion of anteiso-branched FA over the iso-branched FA, indicating an increase of the membrane fluidity. In levofoxacin cells, it was observed a fluctuation in the amount of MUFA (firstly decreased and then increased) and the SSFA increased over the time of the assay, indicating a decrease of the membrane fluidity. For teicoplanin cells, slightly alteration was observed, it was showed a fluctuation of the BSFA and the MUFA increased over time, which indicates a trend to maintain the flexibility. In the case of thioridazine cells, the MUFA decrease, in general the BSFA increased and the polyunsaturated fatty acids (PUFA) as well, this data

![Image](http://example.com/image1.png)

![Image](http://example.com/image2.png)
Figure 9 – Lipid composition of *M. vaccae* cells previously adapted to Aniosrub grown in the presence of ½ of the MIC of the EPIs and levofloxacin, and ¼ of the MIC of teicoplanin. The fatty acids are grouped accordingly to their type: MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids), SSFA (straight-chain fatty acids), BSFA (branched-chain fatty acids), 10-methyl fatty acids, and other fatty acids. The ‘Aniosrub’ represents the control of the assay where the cells were only exposed to the disinfectant.

Figure 10 – Lipid composition of non-adapted *M. vaccae* cells previously adapted to Aniosrub grown in the presence of ½ of the MIC of the antibiotics and EPIs. The fatty acids are grouped accordingly to their type: MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids), SSFA (straight-chain fatty acids), BSFA (branched-chain fatty acids), 10-methyl fatty acids, and other fatty acids. The ‘Aniosrub’ represents the control of the assay where the cells were only exposed to the disinfectant.
indicates a reduction in the flexibility of the cellular membrane. Moreover, the expression of PUFA might indicate de novo synthesis of fatty acids, and this is only possible when bacterial cells are growing [16]. Finally, in omeprazole cells condition, it was observed the highest amount of BSFA of the assay, and together with MUFA had suffered fluctuations during the assay. It is also important to mention that the most part of the BSFA were anteiso-branched FA, with a great contribution of 13:0 anteiso, indicating an increase of the fluidity of the cellular membrane.

Regarding to the FA alterations of non-adapted cells to Aniosrub, shown in Figure 10, it was observed that in all the cases, although some fluctuation, the trend it was to the decrease MUFA over time. In all the cases, it was also showed some amount of PUFA. In control cells, it was also observed the increase of the BSFA with higher proportion of iso-branched FA. In the case of the levofloxacin cells, the BSFA and SSFA suffered a slight fluctuation over the time. For teicoplanin and thioridazine cells, it was observed an increase in SSFA and at late stage of the growth, and the proportion of other FA increased, namely the 17:0 cyclo w7c fatty acid. In omeprazole cells, the SSFA also suffered fluctuation with a trend to increase in the late stage of the growth. Thus, the fluidity of the membrane was reduced in all the cases.

It is also important to refer that either in adapted M. vaccae cells to Aniosrub or non-adapted cells exposed to Aniosrub, it was showed the 10:0 fatty acid in all the conditions tested, it is thought that this fatty acids is produced in response to disinfectants, since it was only showed even when cells were only in presence of the disinfectants, especially in presence of Aniosrub.

Similarly to it was seen in gel alcohol exposure, also in the exposure to Aniosrub, it was verified that non-adapted cells to Aniosrub survived better than the adapted cells. For adapted cells to Aniosrub simultaneously exposed to omeprazole, could be suggested cross-resistance between the two compounds. However, after ca 45 h of growth, the cells started to decline, so other adaptive mechanisms might have been involved in this case. For non-adapted cells to Aniosrub, the omeprazole seemed to have had an initial inhibitory effect upon the growth, and thioridazine seemed have no effect upon the possible existent efflux pumps or it was not activated at all, in the latter situation means that other mechanisms have been involved in the response observed.

IV. CONCLUSIONS

In the adaptation of M. vaccae cells to the disinfectants, gel alcohol and Aniosrub, it was observed that mycobacterial cells adjusted the composition and the proportions of their fatty acids of the cellular membrane to reduce the fluidity. It was also showed that in response to the disinfectants the bacterial cells performed cells aggregation or showed a more elongated form.

The antibiotics showed a better efficacy over the M. vaccae cells adapted to disinfectants. Since, apart from the non-adapted cells exposed simultaneously to Aniosrub and levofloxacin, for all the other non-adapted mycobacterial cells exposed simultaneously to antibiotics it was showed growth. In the latter case the bacteria might have activated efflux systems, since levofloxacin belongs to fluoroquinolones and efflux pumps have already been described to extrude these compounds in M. tuberculosis and M. smegmatis. Nevertheless, other type of adaptive mechanisms might be responsible for this result.

Furthermore, M. vaccae cells adapted to the disinfectants when exposed simultaneously to the disinfectant and to thioridazine have no significant growth, which might suggest, that effectively thioridazine worked as EPI, although for non-adapted mycobacterial cells to disinfectant have not showed the same behaviour. In the bacterial cells adapted to disinfectants and exposed to omeprazole might have occurred cross-resistance, and perhaps also with non-adapted cells, since it was observed growth in all the situation containing omeprazole, although in non-adapted cells the growth have been retarded.

In future studies could be tested if mycobacterial cells in the susceptibility test were really dead or if the few that might have survived are able to re-colonized fresh culture media, and to make sure if the mycobacterial cells are using the efflux systems, another studies might be done, such as a fluorometric assay to evaluate the accumulation and extrusion of substrates in real-time. It could also be tested the effect of antibiotics and EPIs upon mycobacterial biofilm. It would be interesting test other classes of antibiotics and EPIs.
REFERENCES


