Characterization of the mechanisms of adaptation of *Mycobacterium* to organic solvents

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Finda mais uma importante etapa, é importante relembrar e homenagear os nossos companheiros de aventuras. Sim, porque a Ciência é, sem dúvida, uma das mais incríveis aventuras e propósitos ao qual nos podemos dedicar. Como todas as aventuras, tem também algumas intempéries, que sem a ajuda de outros demais tornaria as respostas mais difícil de alcançar, e por vezes até, difíceis de compreender.

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ABSTRACT

This work aims to characterize the mechanisms involved in solvent tolerance in *Mycobacterium vaccae*, particularly alterations in the fatty acid composition of cells, cell morphology and properties of the cell surface.

Solvent tolerance was assessed in the presence of toluene, ethanol, glycerol and MTBE (methyl *tert*-butyl ether), by comparison of the different degrees of inhibition on cellular growth obtained according to the concentration of solvent tested. Cells were able to tolerate higher concentrations of glycerol (20%) and ethanol (5%) than MTBE (1%) and toluene (0.1%) without displaying a very accentuate degree of inhibition, and when studying the differences in the fatty acid composition of cells it was possible to see that the major difference was in the 16:1 w6c FA, whose production was delayed or inhibited in the most toxic solvents tested and also on the highest concentrations tested.

In order to determine if *M. vaccae* can use these solvents as carbon and energy sources, growth in mineral medium supplemented with 0.25% and 1% (v/v) of the different solvents tested was disclosed. Cells were able to grow both on ethanol and glycerol, and to a lesser extent in MTBE. Ethanol and MTBE were chosen to conduct further adaptation experiments, by adding pulses of 5% and 1% solvent, respectively, and fatty acid (FA) composition, zeta potential and cell morphology were analyzed. It was possible to verify that cells tend to increase the saturation degree in the presence of the solvents, and different morphologies were observed in MTBE and ethanol-adapted cells by fluorescence microscopy. Also, the zeta potential of cells was different, becoming more positive in MTBE-grown cells (-33.1 mV) and more negative in ethanol-grown cells (-45.9 mV).

Minimum inhibitory concentration (MIC) of efflux pump inhibitors (EPIs) and antibiotics was determined for ethanol and MTBE-adapted cells and compared with the MIC values obtained for non-adapted cells. Previous reports suggested that solvent-adapted cells increased the resistance towards antibiotics, but few studies were performed in Gram-positive bacteria, and none, to our knowledge, concerned mycobacteria. This report shows that cells adapted to ethanol and MTBE did not increase the MIC of levofloxacin and teicoplanin, suggesting that cells do not display cross-resistance between solvents and antibiotics. Nonetheless, in the presence of the EPIs thioridazine and omeprazole, the MIC was higher in adapted cells when compared with non-adapted cells.

These results point to the fact that mycobacteria present different degrees of tolerance towards different solvents.

Keywords: Solvent tolerance; *Mycobacterium vaccae;* adaptation; fatty acid profile; antibiotics; efflux pump inhibitors.

RESUMO

Este trabalho pretende caracterizar os mecanismos envolvidos em tolerância a solventes, em *Mycobacterium vaccae*, particularmente alterações na composição dos ácidos gordos das células, morfologia e propriedades da superfície celular.

A tolerância a solventes foi analisada na presença de tolueno, etanol, glicerol e MTBE (éter metil-terc-butílico), pela comparação dos diferentes graus de inibição obtidos de acordo com a concentração de solvente testada. As células foram capazes de tolerar melhor glicerol (20%) e etanol (5%) do que MTBE (1%) e tolueno (0.1%) sem demonstrarem um elevado grau de inibição, e aquando do estudo das diferenças na composição dos ácidos gordos foi possível verificar que a maior diferença reside no ácido gordo 16:1 w6c, cuja produção foi afectada ou inibida nos solventes mais tóxicos testados, dependendo da concentração testada. Para determinar se *M. vaccae* consegue utilizar estes solventes como fonte de carbono, as células foram crescidas em meio mineral suplementado com 0.25% e 1% (v/v) dos diferentes solventes testados. As células foram capazes de crescer em etanol e glicerol, e numa menor extensão em MTBE. Etanol e MTBE foram escolhidos para conduzir experiências de adaptação celular, mediante a adição de pulsos de solvente de 5% e 1%, respectivamente, sendo analisada a composição dos ácidos gordos, o potencial zeta e a morfologia das células. Foi possível verificar que as células tendem a aumentar o grau de saturação na presença dos solventes, e foram observadas diferentes morfologias tanto em células adaptadas a MTBE como em células adaptadas a etanol, através de microscopia de fluorescência. O potencial zeta também foi alterado, sendo mais positivo em células crescidas em MTBE (-33.1 mV) e mais negativo em células crescidas em etanol (-45.9 mV).

A concentração mínima inibitória dos inibidores de bombas de efluxo e antibióticos foi determinada para células adaptadas a etanol e a MTBE, e comparada com os valores obtidos para células não-adaptadas. Outros estudos sugerem que células adaptadas a solventes aumentam a resistência a antibióticos, mas poucos foram conduzidos em bactérias Gram-positivas, e nenhum, que seja do nosso conhecimento, foi realizado em micobactérias. Este trabalho conclui que células adaptadas a etanol e MTBE não têm uma concentração mínima inibitória maior relativamente à levofloxacina e à teicoplanina, sugerindo que as células não adquiriram resistência cruzada entre solventes e antibióticos. No entanto, na presença dos inibidores de bombas de efluxo tioridazina e omeprazole, a concentração mínima inibitória é maior em células adaptadas a solventes do que em células não adaptadas.

Estes resultados demonstram que as micobactérias têm diferentes graus de tolerância a diferentes solventes.

Palavras-chave: Tolerância a solventes; *Mycobacterium vaccae*; adaptação; perfil de ácidos gordos; antibióticos; inibidores de bombas de efluxo.

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LIST OF ABBREVIATIONS

MTBE – Methyl *tert*-butyl ether;

MIC – Minimum inhibitory concentration;

EPI – Efflux pump inhibitor;

TEICO – Teicoplanin;

LEVO – Levofloxacin;

THIO – Thioridazine;

OME – Omeprazole;

CLSI – Clinical and Laboratory Standards Institute**;**

FAMEs – Fatty acid methyl esthers;

GC – Gas chromatography;

MH – Mueller-Hinton;

DMSO – Dimethyl sulphoxyde;

rpm – Rotations per minute;

OD_{λ} - Optical density at wavelength λ;

ATP – Adenosine triphosphate;

RND – Resistance nodulation cell division family;

SMR – Small multidrug resistance family;

MFS – Major facilitator superfamily;

ABC – ATP-binding cassette family;

EPS – Extracellular polymeric substances**;**

NTM – Non-tuberculous mycobacteria;

MDR – Multidrug resistance;

FA – Fatty acid;

MUFA – Monounsaturated fatty acid;

PUFA – Polyunsaturated fatty acid;

SSFA – Straight-chain saturated fatty acid;

BSFA – Branched-chain saturated fatty acid;

BTEX – Benzene, toluene, ethylbenzene, xylene;

AST – Antimicrobial susceptibility testing;

MH – Mueller-Hinton media.

1. Introduction

Gram-positive solvent-tolerant bacteria can be very useful in bioremediation of polluted environments and in non-aqueous biocatalysis (Torres, Pandey, & Castro, 2011). Since some of the most commercially interesting substrates are water-insoluble, the establishment of a biphasic organic-aqueous system, in which the substrate is in the organic phase and provided to cells in the aqueous phase is a good solution in order to overcome this problem (Andersson & Gerdal, 1990; de Carvalho et al., 2004; de Carvalho & da Fonseca, 2002; Sardessai & Bhosle, 2004). This way, solvents can act as a reservoir for water-immiscible substrates and also as a sink for toxic products, thus protecting cells from their toxicity (de Carvalho & da Fonseca, 2002; Hamada et al., 2008).

Solvents are a part of several industries and sometimes end up reaching the environment and some of them have been considered as emerging pollutants. Benzene, 1,2-dicloroethane, dichloromethane, hexachlorobutadiene, trichlorobenzenes and trichloromethane (chloroform) are on a list of 48 hazardous substances, along with some PAH's, flame retardants, pesticides and biocides, under the European Commission Water Framework Directive (2000/60/EC) ("Directive 2000/60/EC of the European Parliament and of the Council," 2000). The global demand for solvents has been continuously increasing and covers a wide range of industrial applications. This market can be mainly segmented into five types of products, such as hydrocarbons, alcohols, esters, ketones, chlorinated solvents. Alcohol based solvents represent the largest product segment and it is expected to be one of the fastest growing solvent market from 2014 to 2020 ("Solvent Market Analysis By Product (Hydrocarbons, Alcohols, Esters, Ketones, Chlorinated) By Application (Printing Inks, Paints And Coatings, Pharmaceuticals, Adhesives & Cosmetics) And Segment Forecasts To 2020," n.d.)

Some organic solvents are classical antimicrobial agents, like alcohols, phenols and aromatics. Aromatics have been particularly used as fuels, industrial solvents and as starting materials for chemical processes (Sikkema, de Bont, & Poolman, 1995). Some organic solvents have been present in the environment as a result of natural biosynthesis, although at low concentrations, and can be mineralized by microbial communities (Isken & de Bont, 1998).

The Corynebacteriaceae is the taxonomic family that harbors most Gram-positive bacteria with xenobiotic biodegradability capacity, including the highly efficient biodegraders *Gordonia*, *Rhodococcus* and *Mycobacterium* (Bell, Philp, Aw, & Christofi, 1998; Willumsen & Karlson, 1997). These communities acquire resistance or tolerance to xenobiotics and some cases of pathogenicity to humans often arise. This is where the problematic of cross-resistance between organic solvents and antibiotics begins. If microorganisms had already a first contact with organic solvents in their natural environments, it would probably trigger mechanisms leading to an improved resistance regarding antibiotics.

1.1 Bacterial tolerance and resistance

The first report of an organic solvent tolerant bacterium was presented in 1989 on a *Pseudomonas putida* IH-2000 strain able to endure high toluene concentrations (more than 50% v/v). This strain was unable to use it for growth, and the bacterial response to other solvents enabled the establishment of a possible partial relation between solvent toxicity and polarity (Inoue & Horikoshi, 1989).

Organic solvents can be extremely toxic to cells, even at concentrations as low as 0.1% (v/v), since they can partition and accumulate in the bacterial cell (Isken & de Bont, 1998). A solvent is considered toxic when the metabolic activity of cells is reduced to less than 50% of the metabolic activity when compared to cells grown in the absence of solvent (Vermue et al., 1993). Toxicity can be predicted based on the *log* P value, which is a direct measure of solvent polarity and can be determined experimentally using a system *n*-octanol:water (Laane, Boeren, Vos, & Veeger, 1987). Polar solvents have a lower *log* P value (< 2) and thus they are considered to be more toxic (Sardessai & Bhosle, 2002), and non-suitable for biocatalytic systems, since they strongly distort the water-biocatalyst interaction, leading to the inactivation or denaturation of the biocatalyst. Solvents with a *log* P value between 2.0-4.0 tend to partition mainly to the cytoplasmic membrane (Ramos et al., 2002), and are weak water distorters, having an unpredictable effect in the biological activity of the biocatalyst, whilst solvents with a *log* P value higher than 4 are lipophilic, and even though they accumulate in the cellular membranes, they will not be toxic due to their poor water solubility, not distorting the water coat surrounding the biocatalyst, being considered biocompatible (de Carvalho et al., 2004; Laane et al., 1987; Sardessai & Bhosle, 2004).

A direct relation between *log* P value and cellular response is not always valid. In the *Bacillus cereus* strain R1, solvent toxicity could not be calculated based on the *log* P value of the solvents, since aliphatic alcohols with high *log* P values inhibited the growth of cells (Matsumoto, de Bont, & Isken, 2002). Cell tolerance was shown to be higher in water miscible solvents with lower *log* P values than those with higher values in *R. erythropolis* and *Mycobacterium* sp., showing that the *log* P criterion may fail to predict the solvent toxicity at small range scale. This might be associated with the low permeability of both organisms to hydrophilic solutes (de Carvalho et al., 2004).

Solvent toxicity (Figure 1) is not only related to the solvent hydrophobicity, but also to the solvent molecular structure (Vermue et al., 1993) and the characteristic of the cell membranes (Bruce & Daugulis, 1991). Each bacterium is known to possess a strain-specific level of tolerance to organic solvents, either by genetic determination or environmental factors (Kobayashi, Yamamoto, & Aono, 1994). Another characteristic of organic solvents is their capability of causing molecular and/or phase toxicity in biphasic systems. Molecular toxicity relates to the molecules of solvent present in the aqueous phase; phase toxicity is caused by the direct cell-solvent contact when a distinct second phase is present. Phase toxicity is predicted by the *log* P value, and might result from the extraction of cell wall components and nutrients or limited availability of nutrients. Molecular toxicity may cause enzyme inhibition, protein denaturation and membrane modification (Vermue et al., 1993). In systems with water-miscible solvents, cells are in the aqueous phase where the solvent accumulates, and they are only subjected to molecular toxicity, but, in systems with water-immiscible solvents, cells can be

located in the aqueous:organic interface or in the organic phase, being exposed to both types of toxicity (de Carvalho & da Fonseca, 2005). Vermuë *et. al.,* suggest that the possible differences between Gram-positive and Gram-negative bacteria may be related to the differences in response to phase and molecular toxicity, since solvent toxicity depends on the presence of a second phase and on the concentration of solvent in the aqueous phase. Assuming the critical membrane theory to be valid, it led the authors to conclude that differences between the two types of bacteria could not be justified by differences in response to molecular toxicity (Vermue et al., 1993).

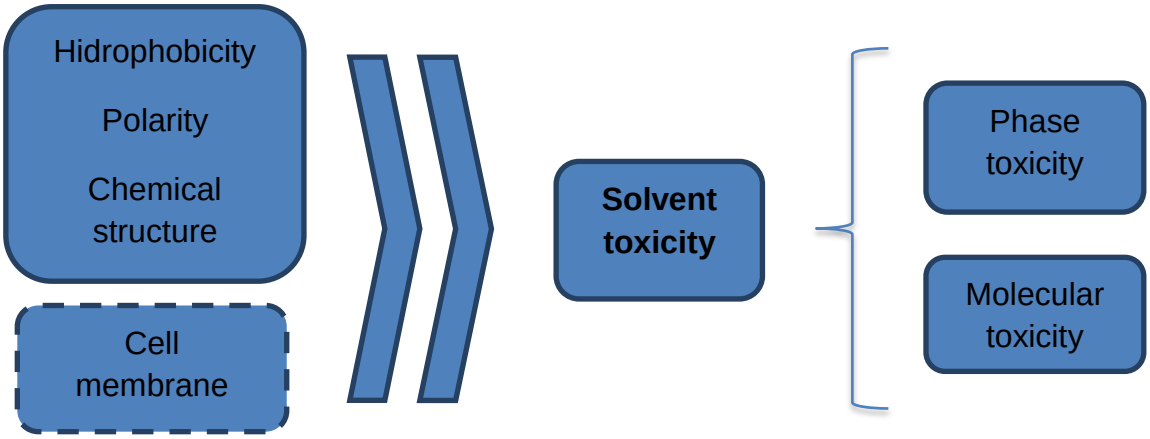


Figure 1 - Mechanisms of solvent toxicity and types of toxicity exerted by organic solvents.

1.2 Bacterial adaptation mechanisms

The accumulation of toxic compounds in bacterial membranes can cause deleterious permeability effects, compromising cellular integrity and survival. Bacteria have developed a wide range of mechanisms to cope with toxic organic compounds, being the cytoplasmic cell membrane the most important protective barrier against toxic compounds (Unell, Kabelitz, Jansson, & Heipieper, 2007).
Bacteria use a lot of mechanisms to overcome solvent stress. Some mechanisms include extrusion of solvents through efflux pumps, modification of phospholipids headgroups, vesicle formation, adjustments in the fatty acid composition of cells, and modifications in the biosynthesis rate of phospholipids. These mechanisms have been extensively reviewed for Gram-negative bacteria and reports about tolerance mechanisms in Gram-positive bacteria have captured more attention in the last two decades (Bustard, Whiting, Cowan, & Wright, 2002; de Carvalho & da Fonseca, 2005; de Carvalho, 2012; de Carvalho et al., 2004; de Carvalho, Fatal, Alves, & da Fonseca, 2007; de Carvalho, Parreño-Marchante, Neumann, da Fonseca, & Heipieper, 2005; Matsumoto et al., 2002; Segura, Hurtado, Rivera, & Lazaroaie, 2008; Zhang, Zhang, Cheng, Yao, & Chen, 2013) (Table 1).

Some mechanisms are common to both types of bacteria while others seem to be specific, such as the *cis-trans* isomerization, which has been reported in Gram-negative bacteria.

The hypothesis that Gram-negative bacteria are more fitted to overcome solvent toxicity than Gram-positive is now considered an old idea, since recent reports of remarkable solvent tolerance comprising Gram-positive bacteria have emerged. The *Brevibacillus agri* strain 13 was able to tolerate 5 and 20% (v/v) ethyl and butyl acetate, with *log* P values of 0.86 and 1.90, respectively (Kongpol et al., 2009). *R. erythropolis* DCL14 was able to survive in 50% (v/v) of the extremely toxic toluene (de Carvalho et al., 2007), pointing out to the ability of some Gram-positive strains to overcome very aggressive organic solvent conditions. The next section will focus on the many adaptation mechanisms occurring particularly in Gram-positive bacteria (Figure 2), with particular emphasis on the mechanisms tackled throughout the experimental work.

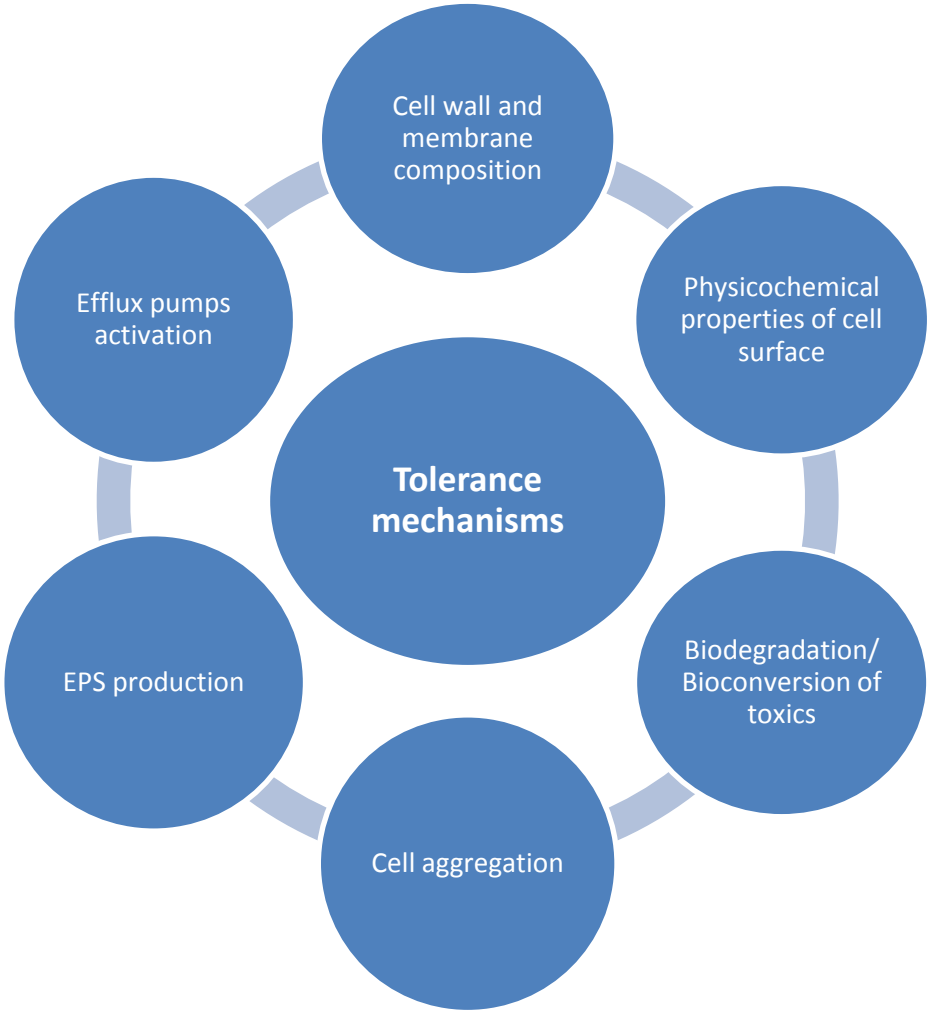


Figure 2 - Adaptation mechanisms reported in Gram-positive bacteria related with solvent toxicity.

Table 1 - Previous reports concerning Gram-positive bacteria with remarkable solvent-tolerance.

Strain	Solvent tolerance	Reference
Bacillus pallidus	Use of acetone and isopropanolol as sole carbon source at 60ºC.	(Bustard et al., 2002)
Bacillus cereus strain R1	Growth in the presence of a separate phase of toluene or other hydrocarbons, but not aliphatic alcohols.	(Matsumoto et al., 2002)
Bacillus DS-994	Growth in the presence of 5% (v/v) benzene and 10% (v/v) toluene.	(Moriya & Horikoshi, 2000)
Bacillus subtilis GRSW2-B1	Tolerance to a range of organic solvents at high concentration (5%v/v), with remarkable tolerance in particular to butanol and alcohol groups.	(Kataoka, Tajima, Kato, Rachadech, & Vangnai, 2011)
Brevibacillus agri strain 13	High level of tolerance toward organic solvents at 5% (v/v), with a broad range of log Pow values, such as n-tetradecane (7.2), cyclohexane (3.2), n-pentanol (1.3), n-butanol (0.84), iso-butanol (0.77), butyl acetate (1.9) and ethyl acetate (0.86) as well as a fair level of tolerance toward toluene (2.5), o-xylene (3.1) and benzene (2.0).	(Kongpol et al., 2009)
Mycobacterium cosmeticum byf-4	Biodegradation of benzene, toluene, ethylbenzene and o-xylene both individually and as a composite mixture.	(Zhang et al., 2013)

Mycobacterium aurum L1	Used in a mixed culture with *Xanthobacter autotrophicus* GJ10, it can simultaneously remove and mineralize vinyl chloride and 1,2-dichloroethane. Growth in vinyl chloride as sole carbon and energy source.	(Hartmans & De Bont, 1992; Hartmans, Kaptein, Tramper, & de Bont, 1992)
Mycobacterium sp. PH-06	Use of 1,4-dioxane as sole carbon and energy source. Transformation of structural analogues such as 1,3-dioxane, cyclohexane and tetrahydrofuran when pre-grown with 1,4-dioxane as the sole growth substrate.	(Kim, Jeon, Murugesan, Kim, & Chang, 2009)
Mycobacterium vaccae JOB5	Both propane- and 1-butanol-induced JOB5 and *Rhodococcus jostii* RHA1 were able to degrade 1,4-dioxane, TCE, and mixtures of 1,4-dioxane/TCE.	(Hand, Wang, & Chu, 2015)
Mycobacterium sp. LB501T	Use of solid anthracene as sole carbon source.	(Wick, Ruiz de Munain, Springael, Harms, & De, 2002)
Mycobacterium E20	Utilization of ethylene, ethylene oxide and ethane as the sole source of carbon and energy for growth.	(de Bont, Attwood, Primrose, & Harder, 1979)
Mycobacterium spp. strains TA5 and TA27	Degradation of trichloroethylene and 1,1,1-trichloroethane. Both bacteria could cometabolically degrade dichloromethane, chloroform, 1,1-dichloroethane, 1,2-dichloroethane, 1,1,1-TCA, 1,1,2-TCA, 1,1,1,2-tetrachloroethane, 1,1,2,2-tetrachloroethane, 1,1-dichloroethylene, *cis*-1,2-dichloroethylene, *trans*-1,2-dichloroethylene, and TCE with ethane as a carbon source.	(Hashimoto, Iwasaki, Nakasugi, Nakajima, & Yagi, 2000)

Rhodococcus sp. 33	Growth in benzene in both vapour and liquid phases in batch and continuous cultures.	(Paje, Neilan, & Couperwhite, 1997)
Rhodococcus opacus B-4	Survival in essentially non-aqueous environments with less than 1%(w/v) water.	(Yamashita et al., 2007)
Rhodococcus erythropolis DCL14	Survival in 50% (v/v) toluene after 15 minutes of contact. Growth in 15% (v/v) methanol, 20% (v/v) ethanol, 5% (v/v) propanol and dodecanol, 2% (v/v) butanol and 1% (v/v) cyclohexanol. Growth in 1% (v/v) of different *n*-alkanes (C5-C16) as sole carbon and energy source. Biodegradation of C5–C16 hydrocarbons and C1–C12 alcohols as sole carbon and energy sources at 15 and 28ºC in the presence of salt.	(de Carvalho & da Fonseca, 2005; de Carvalho et al., 2007; de Carvalho, Marques, Hachicho, & Heipieper, 2014; de Carvalho et al., 2005; de Carvalho, Wick, & Heipieper, 2009)

1.2.1 Alterations at the cell wall and membrane composition

All adaptive mechanisms involved in the maintenance of a constant membrane fluidity are due to a phenomenon known as “homeoviscous adaptation” (Sinensky, 1974). Organic solvents act firstly by leading to a specific permeabilization of the cell membranes, as a result of a damaged cytoplasmic membrane (Isken & de Bont, 1998). Hydrophobic organic solvents intercalate into biological membranes, weakening lipid interaction and disturbing the membrane structure (Sikkema, de Bont, & Poolman, 1994). Other studies showed that in the presence of solvents, the membrane surface area increases and a passive flux of ions and protons across the membrane takes place (Leão & Van Uden, 1984; Sikkema et al., 1994) dissipating the proton motive force and affecting both the proton gradient and the electrical potential, ultimately leading to a decrease in the energy status of the cell (Cartwright et al., 1986). The ATP synthesis can also be damaged, and both ATPase and other proteins, either involved in the energy-transducing process or embedded in the membrane are also affected (Bowles & Ellefson, 1985; Uribe, Rangel, Espinola, & Aguirre, 1990). All these mechanisms end up having consequences at the level of the membrane fluidity (Sikkema et al., 1994).

Bacteria maintain constant membrane fluidity mainly by changing the degree of saturation of the fatty acids. Increasing the amount of saturated fatty acids causes a decrease in the membrane fluidity, whereas the incorporation of unsaturated fatty acids results in an increase in membrane fluidity. This change in fatty acid composition is strictly associated to cell growth, because it requires *de novo* synthesis. If growth is abolished, fatty acid biosynthesis and modification of the saturation degree are blocked (de Carvalho et al., 2005). Under growth-limiting conditions, lipid biosynthesis is inhibited due to stringent response regulation. Under these conditions, only enzymatic modifications may occur, such as *cis-trans* modification, in Gram-negative bacteria. This short-term response is triggered when hydrophobic chemicals are present and fatty acid biosynthesis is inhibited, leading to enzymatic modifications in the the ones synthesized prior to the addition of the toxics (Murínová & Dercová, 2014). In *R. erythropolis*, a fast post *de novo* synthesis modification of saturated and monounsaturated fatty acids to synthesize polyunsaturated fatty acids was reported when cells were subjected to salt stress, suggesting a change in fatty acid composition due to an enzymatic mechanism (de Carvalho et al., 2014).

Gram-positive bacteria rely also on a high proportion of *iso*- and *anteiso*-branched fatty acids to adapt their fluidity. These fatty acids derive from valine, leucine and isoleucine precursors resulting in *iso*-branched-even-chain, *iso*-branched-odd-chain and *anteiso*-branched-odd-chain fatty acid species, with different structures and physico-chemical properties (Kaneda, 1991). A change from *iso* to *anteiso* configuration results in an increase of membrane fluidity, associated with the steric differences between them. In the psychotroph *Arthrobacter clorophenolicus* A6 grown on phenols, a decrease in the *anteiso/iso* ratio was registered at

higher growth temperatures, resulting in a more rigid membrane, counteracting the conjugated effect of both temperature and solvent. Under psycrophilic conditions, the opposite behavior was observed: bacteria showed an increase of *anteiso* fatty acids at lower temperature, in order to decrease the membrane viscosity following solvent exposure (Unell et al., 2007).

Phospholipids headgroups may also change after solvent exposure. Along with cardiolipin, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol and phosphatydilinositol mannosides are considered to be the major phospholipids of mycobacteria (Murphy & Perry, 1987). In *n*-hexadecane-grown *M. convolutum* R22, phosphatidylethanolamine and phosphatydilinositol mannosides were the major phospholipids present (Murphy & Perry, 1987), whilst following growth on propane, phosphatidyl-L-serine, cardiolipin and phosphatidylethanolamine were the major phospholipids present in *Mycobacterium vaccae* (Vestal & Perry, 1969).

1.2.2 Modifications of the physicochemical properties of the cell surface

Bacteria may adapt to hydrophobic chemicals in their surroundings via a modification of their cell surface characteristics, such as charge and hydrophobicity (Wick et al., 2002). Cell surface characteristics are related to the ability of bacterial cells to access water-immiscible substrates in two-liquid phase processes and to adapt to hydrophobic compounds. A change in cell surface hydrophobicity when incubated in the presence of organic solvents was observed in Gram-negative bacteria and also described in Gram-positive bacteria, being this mechanism known as hydrophobic switch (Torres et al., 2011). The cell surface charge of bacteria is a physicochemical property that depends on the structure of their cellular envelope and on several types of interactions with its surrounding environment. Under physiological conditions, the net surface charge of bacteria is usually negative, and it can change after the modification of the environmental conditions (Ayala-Torres, Hernández, Galeano, Novoa-Aponte, & Soto, 2013; de Carvalho et al., 2009).

One of the primary methods for measurement of the cell surface charge is the zeta potential determination, which can be estimated by the electrophoretic mobility of cells. This parameter depends on the pH, ionic strength, temperature, and even on the metabolic status, viability and number of cells, as reported very recently in a study aiming to differentiate virulent and non-virulent mycobacteria based on their surface charge (Ayala-Torres et al., 2013).

In *M. frederiksbergense* LB501T it was shown that the cell surface hydrophobicity was positively correlated to the aqueous solubility of the growth substrate used. The higher the proportion of anthracene in a glucose mixture, the higher the cell surface hydrophobicity and more hydrophobic the mycolic acids produced by the cells (Wick, Pasche, Bernasconi, Pelz, & Harms, 2003). The surface hydrophobicity of *R. erythropolis* IBBPo1 cells was altered when

these were grown on either alkanes or aromatics. Alkanes were less toxic and induced an increase in cell surface hydrophobicity, whereas the opposite was observed with aromatics. Low hydrophobicity is seen as a defensive mechanism, which repels organic solvents from the cell surface, and thus prevents from damaging cell membrane (Stancu, 2014). Strong evidence between cell-surface hydrophobicity and cellular affinity for organic solvents was reported, since more hydrophobic cell-surfaces have more affinity towards organic solvents (Yamashita et al., 2007).

1.2.3 Cell aggregation

The hydrophobicity of bacterial cell envelope is directly related to the ability of cells to bind toxic compounds (Torres et al., 2011). Cells from the *Mycobacterium* genus form large cellular aggregates as a consequence of their cellular hydrophobicity (Figure 3). This hydrophobicity was shown to depend not only on the lipid content, but also on the fatty acid composition of cells and on the carbon source available. The presence of paraffins in these cells also seems to be directly involved in mycobacterial cell aggregation (Borrego, Niub, & Espinosa, 2000).

Arthrobacter simplex and *R. erythropolis* only formed aggregates in the presence of low *log* P solvents, whereas in *Mycobacterium*, aggregation occurred even in high *log* P solvents (de Carvalho & da Fonseca, 2004). This behavior was also described in *Mycobacterium sp*. and *R. erythropolis* cells in the presence of butan-1-ol (de Carvalho et al., 2004), and in *Mycobacterium sp*. NRRL B-3805 exposed to bis(2-ethylhexyl) phthalate (BEHP) changes in cell size, circularity, elongation and aggregation were assessed using scanning electron microscopy (Angelova et al., 2006).

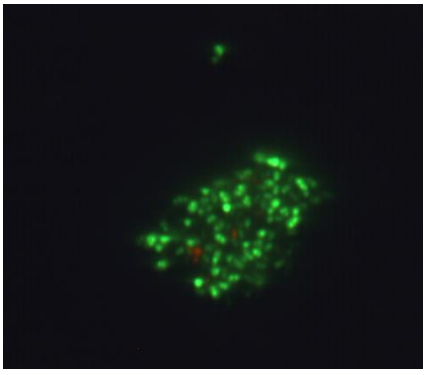


Figure 3 - Cell aggregation in *M.vaccae* as a response to solvents.

1.2.4 Activation of efflux pumps

Bacteria rely on the use of efflux pumps to extrude antibiotics and chemicals, conferring these cells drug resistance (Balganesh et al., 2012). Efflux pumps can be very important in extrusion of molecules, acting by decreasing the solvent concentration inside the cell (Torres et al., 2011). Efflux pumps can be divided into five major families: the resistance nodulation cell division family (RND), the small multidrug resistance family (SMR), the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion family (MATE) and the ATP-binding cassette family (ABC) (Fernandes, 2003) (Figure 4). Transporters from the ABC family contain a highly conserved cytoplasmic nucleotide binding domain, allowing them to use energy derived from the ATP hydrolysis to actively transport compounds against the concentration gradient. MFS, SMR, MATE and RND rely on the electrochemical gradient to exchange outside protons for drug molecules (Markham & Neyfakh, 2001).

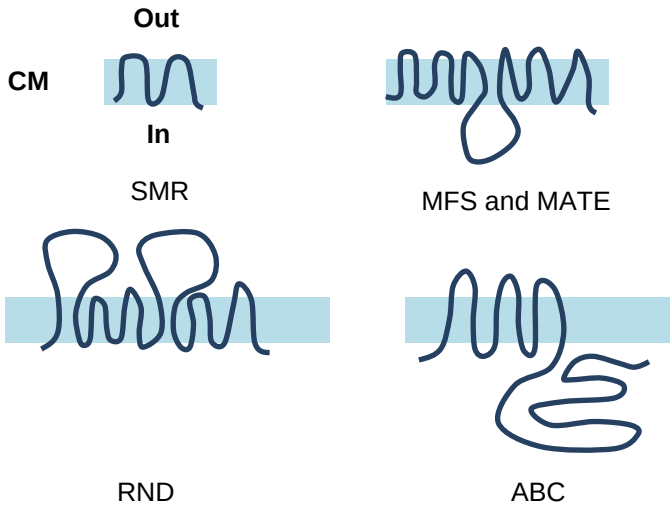


Figure 4 – Model of efflux pumps corresponding to SMR, MFS, MATE, RND and ABC family. Adapted from (Kaatz, 2002)**. In and out refer the inside and the outside of the cellular membrane (CM).**

The role of efflux pumps in solvent extrusion has been widely described as a tolerance mechanism in species from the *Pseudomonas* genus (Isken, 1996). In *Pseudomonas putida* DOT-T1E cells, efflux pump mexB activity was studied for toluene and 1,2,4-[14C]trichlorobenzene (Ramos, Duque, Godoy, & Segura, 1998) and in *P. aeruginosa* MexA-MexB-OprM was shown to contribute to high intrinsic antibiotic resistance.

In Gram-positive bacteria, a wide variety of multidrug efflux pumps were described, but the involvement of efflux pumps and their characterization in organic solvent efflux is still poorly discriminated, and only few reports are known to public. In *B. oleronius,* the involvement of efflux pumps as a mechanism of resistance against organic solvents was confirmed using efflux pump inhibitors (Edward, Melchias, Visvanathan, Soni, & Ajithan, 2012). A hydrocarbon pumping-activity was also reported in *B.cereus* R1 in the presence of toluene (Matsumoto et al., 2002).

To our knowledge, no study involving efflux pumps of mycobacteria was addressed to solvent tolerance. Genome sequences of mycobacteria have disclosed the existence of multiple putative efflux pumps and the emergence of reports concerning the involvement of mycobacterial efflux pumps in antibiotic resistance has gained attention. The LfrA protein of *M. smegmatis* was the first efflux pump to be described in the *Mycobacterium* genus and was shown to be responsible for acquired resistance to fluoroquinolones. Also, in *M. fortuitum,* the Tap multidrug efflux pump was known to confer low-level resistance to aminoglycosides and tetracyclines, and led to the identification of the homolog Rv1258c in *M. tuberculosis* (Piddock, 2006; Viveiros, Leandro, & Amaral, 2003). The P55 efflux protein was also shown to confer resistance to these two classes of chemicals (Silva et al., 2001). *M. avium* was shown to possess an intrinsic efflux pump system involved in erythromycin resistance. In the presence of amikacin and ethambutol, an increase in susceptibility was caused by the presence of the efflux pump inhibitors thioridazine and carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Rodrigues et al., 2008).

Efflux pumps activity can be inhibited by efflux pump inhibitors (EPIs), which are known to interfere with the membrane potential of cells, leading to the depletion of the energy necessary to support active transport. In order to be classified as an EPI, a compound has to obey certain requisites (Lomovskaya & Watkins, 2001): 1) it must enhance the activity of multiple substrates of the pump, keeping them inside the cells; 2) it must not show activity on strains lacking the efflux pump; 3) it should decrease efflux of the efflux pump substrates and increase accumulation; 4) the activity of the compound must not affect directly the integrity of the cell membrane and the viability of the bacterial cells, at the concentrations used for efflux inhibition. The protonophore CCCP acts by affecting the energy level of the membrane, dissipating the proton motive force, being thus effective in all efflux pumps that depend on this energy source to operate. The activity of the efflux pump inhibitors leads to the accumulation of the substrate inside the cell, inhibiting the bacterial cell replication (in the case of antibiotics/antibiotic resistance) or enhancing intracellular biotransformations (in the case of organic compounds transformations/degradation and solvent tolerance) (Fernandes, 2003; Torres et al., 2011).

1.3 Mycobacterium

Mycobacterium leprae was the first mycobacteria species to be isolated in 1874 by Hansen (Cook et al., 2009). This genus is commonly divided into slowly and rapidly growing mycobacteria, being the former associated to human diseases and the latter to environmental species. Species from this genus are identified through the Ziehl-Neelsen stain for acid fastness, are aerobic, and usually assume a form of non-motile rods, but they can have mycelium-like growth and branching due to fragmentation. The colonies are usually white or cream colored and rapid growers can have yellow or orange colonies due to the presence of carotenoids (Katoch, 2004). Cultures of mycobacteria are described as being highly heterogeneous due to cell clumping, being possible the co-existence of both dormancy and growth in the same culture. Virulence levels of mycobacteria can also be associated to different colony morphology (Smeulders, Keer, Speight, & Williams, 1999). Colony morphology is due to phase-variable expression of outer membrane or cell wall components, and rough/smooth colonies may be formed due to differences in the types of lipids and polysaccharides present on the cell surface (Smeulders et al., 1999).

Non-tuberculous mycobacteria (NTM) represent the group of saprophytic mycobacteria and colonize a broad range of environments such as soil, water and surgical solutions and are known to cause disease in immunocompromised patients (Katoch, 2004). Recently it was found that tap water is the main infection source (Primm et al., 2004). NTM are important emerging pathogens, due to the growing proportion of population with predisposing conditions. They possess high surface adherence due to their lipid and wax-rich cell wall, influencing biofilm formation and resistance to antibiotics and disinfectants (Briancesco, Semproni, Paradiso, & Bonadonna, 2013). Data from clinical laboratories show that *Mycobacterium avium* complex, *M. gordonae*, *M. xenopi*, *M. kansasii* and *M. fortuitum* are the most frequently isolated species and that new species are emerging from different locations all the time (Briancesco et al., 2013).

1.3.1 Relevance

Mycobacterial species are well known biocatalysts and biodegraders, having a considerable potential in bioremediation of polluted environments (Claudino et al., 2008; Kim et al., 2009; Llanes, Fernandes, Léon, Cabral, & Pinheiro, 2001; Wick et al., 2003). Very recently, a report regarding mycobacteria isolated from the Yellowstone National Park and the Glacier National Park presents mycobacteria with a remarkable capacity to withstand hydrophobic stressors in an extreme ecosystem (Santos, de Carvalho, Stevenson, Grant, & Hallsworth, 2015). But besides their advantages regarding environmental issues, the emergence of new isolated strains from several environments and the recognition of many species from this genus as

opportunistic pathogens, has raised the need of understanding the defense mechanisms that they possess against our own defense strategies. It is imperative to understand how these species can overcome antibiotic treatment and what cellular features they possess in order to do so. Tuberculosis is the world's leading cause of mortality due to a bacterial agent, *Mycobacterium tuberculosis* (Silva et al., 2001) and they can also cause disease in animals, raising economic issues.

1.3.2 Mycobacterial cell wall and cell membrane

Mycobacteria are known to have highly lipidic cell walls, acting as a major barrier against antimicrobial agents. It is structurally more complex than Gram-positive bacteria cell walls, being composed of high molecular weight lipids, the mycolic acids, and an inner region rich in peptidoglycan linked to arabinogalactan (Figure 5). The peptidoglycan is composed by N-glycolymuramic acid instead of N-acetylmuramic acid. The outer region is mainly formed by lipids and waxes, including glycopeptidolipids (only in NTM mycobacteria (Schorey & Sweet, 2008)), trehalose containing lipooligosaccharides, sulpholipids, phthiocerol dimycocerosate and phenolic glycolipids, forming an asymmetric bilayer-like structure (Lambert, 2002). Mycolic acids have 70-90 carbon atoms and represent 60% of the whole cell dry weight. They are responsible for the waxy coat around the polysaccharides, and are covalently linked to the arabinogalactan polymer (Lambert, 2002). These cells also have a wide variety of long-chain saturated (C18-C32) and monounsaturated (<C26) *n*-fatty acids as well as methyl-branched fatty acids (Kolattukudy, Fernandes, Azad, Fitzmaurice, & Sirakova, 1997). The unusual cell wall with low permeability characteristic of mycobacteria is seen as a major drawback in the eradication of mycobacterial diseases.

Mycobacteria have a cell envelope that comprises both characteristics of Gram-positive and Gram-negative bacteria, and a genome-based phylogeny places them in between. This cell envelope is directly related to bacterial virulence (Silhavy, Kahne, & Walker, 2010), being highly rigid and composed by specific mycolic acids (Wick et al., 2003). There is still some discussion whether to consider this cellular feature as a cell envelope or an outer membrane (Hoffmann, Leis, Niederweis, Plitzko, & Engelhardt, 2008).

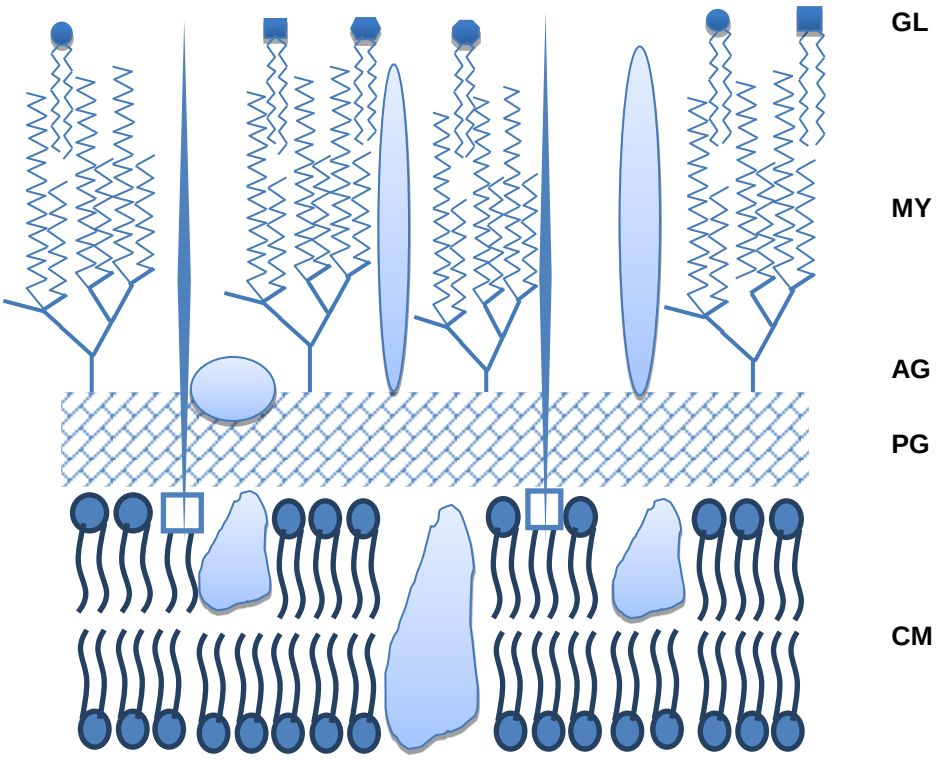


Figure 5 - Model resembling the structure of the cell wall of mycobacteria. Adapted from (Lambert, 2002)**. CM – cytoplasmic membrane; PG – peptidoglycan; AG – arabinogalactan; MY – mycolic acid; GL – glycolipids (several types). The cell wall of mycobacteria contains lipoarabinomannan possibly linked through a phosphatidylinositol lipid (□) to the membrane.**

Hydrophobic antibiotics can still cross the cell wall by diffusion through the hydrophobic bilayer. Hydrophilic antibiotics are thought to use porin channels, giving strength to the view that mycobacteria are composed by an outer membrane similar to the one of Gram-negative bacteria (Lambert, 2002). Some of these porins have already been described in many mycobacteria, such as the MspA of *M. smegmatis*, and Rv0899 and Rv1698 of *M.tuberculosis* (Niederweis, 2003; Siroy et al., 2008).

1.3.3 Mycobacterium vaccae

Mycobacterium vaccae (Figure 6) was described in 1964 by Bönicke and Juhasz, being strongly associated with cattle (Ho et al., 2012). This species is included in the group of rapidly growing mycobacteria and forms yellow-pigmented colonies. It can be found in several environments such as soil, water and milk products (Ho et al., 2012). *M. vaccae* can produce

an immune response against *M. tuberculosis*, being used as a model for tuberculosis immunotherapy, and as an additive to BCG (Chen et al., 2011).

Experiments with mice exposed to *M. vaccae* led to a decrease in anxiety after growth stimulation in certain neural types and serotonin increase, being this species considered beneficial to the central nervous system (Franco et al., 2013). Despite its advantages regarding immune therapies and medicinal applications, it is an opportunistic pathogen and it has been associated to pulmonary and cutaneous infections (Katoch, 2004).

M. vaccae is known to metabolize acetone, cyclohexane, styrene, benzene, ethylbenzene, propylbenzene, dioxane and 1,2-dichloroethylene and possesses a co-oxidative capacity which results in the formation of intermediate molecules more amenable to mineralization due to the degradation of recalcitrant compounds (Burback & Perry, 1993). This is one of the few *Mycobacterium* species known to grow on toluene and it can use acetone as carbon source (Tay et al., 1998).

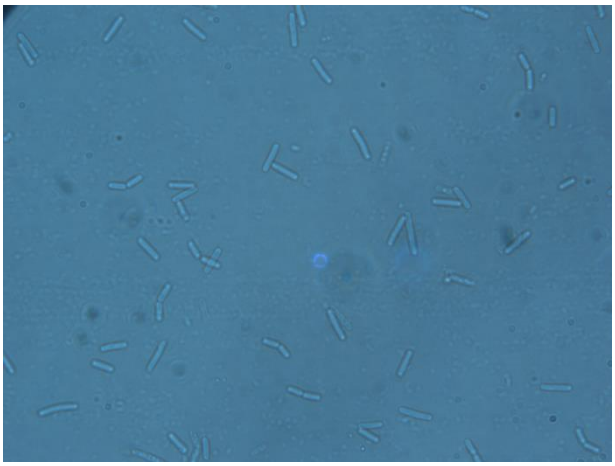


Figure 6- *Mycobacterium vaccae* ATCC 15483 used in the experimental work.

1.4 Relevance of studying the effect of organic solvents in cells

Organic solvents exist widely distributed in the environment, and can be mineralized by bacterial communities. Mycobacteria are known to degrade and transform these compounds, having developed mechanisms to endure their presence. It is then important to study if by contacting with these chemicals, mycobacterial cells are more prone to resist to other type of chemicals such as antibiotics, through common adaptation mechanisms.

1.4.1 Cross-resistance between organic solvents and antibiotics

The role of several efflux pumps in the extrusion of both antibiotics and organic solvents was extensively reviewed by Fernandes *et*. al., (Fernandes, 2003) in Gram-negative bacteria, emphasizing efflux as a cross-resistance involved mechanism and pointing out the need of studying this problematic in Gram-positive organisms, since there are almost no reports regarding these microorganisms. The presence of solvents in the environment together with the abuse of antibiotics and biocide agents may contribute to the propagation of solvent resistant strains (Fernandes, 2003). Cross resistance between organic solvents and between organic solvents and antibiotics was already described for *P. putida* S12, which was more tolerant to ethanol when pre-adapted to supersaturating concentrations of toluene (Heipieper & De Bont, 1994) and more resistant to hydrophobic antibiotics after exposure to toluene. In a study regarding different serotypes of *Salmonella*, the authors were able to validate that cyclohexane-resistant strains of *Salmonella* were more resistant to antibiotics, such as ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nalixidic acid, tetracycline, trimethoprim, cetrimide and triclosan, due to the involvement of AcrAB (Randall, L. P., Cooles, S. W., Sayers, A. R., Woodward, 2001). In Gram-negative negative bacteria, RND efflux pumps were shown to accept both organic solvents and antibiotics as substrates and it was anticipated that higher levels of antibiotic resistance would lead to higher levels of solvent-tolerance, being thus surprising that Gram-positive bacteria, which are more permeable to antibiotics, were also more tolerant to solvent toxicity (Zahir, Seed, & Dennis, 2006).

In *Rhodococcus ruber* previously grown in hydrocarbons, an increased resistance to aminoglycosides, linkosamides, macrolides, β-lactams, and aromatic compounds was reported and correlated with alterations at the level of the membrane fatty acid composition (Kuyukina, B, Rychkova, & Chumakov, 2000). Cells of *B. cereus* strain R1 adapted to toluene were thought to comprise an efflux system able to extrude this compound, but experiments performed with antibiotics showed that this hydrocarbon-pumping is not involved in resistance to antibiotics, since toluene adapted cells did not become more antibiotic resistant towards the antibiotics tested (Matsumoto et al., 2002).

Further studies need to be conducted with different classes of antibiotics and organic solvents, as well as with different microbial species, in order to have a considerable amount of information to drive conclusions on this topic.

1.4.2 Possible common mechanisms of adaptation to solvents and antibiotics

Mechanisms of adaptation to both types of compounds may be associated to general stress mechanisms and multidrug resistance mechanisms, since they are structurally unrelated but can have similar effects on cells, thus triggering similar responses. In a *P. putida* DOT-T1E-18, with a mutation on a RND efflux pump gene TtgABC, a decrease in toluene and antibiotic tolerance was observed. This evidence points out the importance of multidrug efflux pumps in the increase of resistance to all drugs extruded by the pump (Ramos et al., 1998). TetL, a tetracycline resistance determinant involved in efflux mediated antibiotic resistance, was shown to negatively affect the solvent tolerance of *B. subtilis* GRSW2-B1 (Kataoka et al., 2011). The existence of a link between solvent tolerance and multiple drug resistance is very well studied in *E. coli*, being the presence of efflux pumps (acrAB) and of transcriptional activators (*mar*A and *sox*S) one of the main known mechanisms (Randall, L. P., Cooles, S. W., Sayers, A. R., Woodward, 2001). The overexpression of these genes is known to increase resistance to hydrophobic antibiotics. A higher copy number of *mar*A gene is also responsible for higher organic solvent tolerance acquisition (Kobayashi, Kobayashi, Asako, & Aono, 1997).

In *R. ruber* grown in rich media containing gaseous or liquid *n*-alkanes, the cells seemed to increase the resistance to aminoglycosides, lincosamides, macrolides and β- lactams, mainly by the increase of straight-chain saturated fatty acids and by the production of cardiolipin and phosphatidylglycerol, resulting in a decrease of permeability of the cell envelope, preventing the access of antibiotics to the intracellular space (Kuyukina et al., 2000).

Mycobacterial intrinsic drug resistance is mainly associated with active efflux systems and low permeability of the cell wall. In *M. tuberculosis*, 26 complete and 11 incomplete ABC proteins, 20 MFS genes and 15 putative RND family proteins are known. A *Mycobacterium* multidrug resistant protein (MMR) was also found in this genus, belonging to the SMR family, which extrudes a wide range of toxic compounds from the cell (Malkhed, Mustyala, Potlapally, & Vuruputuri, 2014). Other adaptation mechanisms referred in the previous chapters related with organic solvent tolerance might also be implicated in resistance to antibiotics.

1.5 Objectives

The primary objective for this master thesis is to ascertain the different phenotypic changes by which *Mycobacterium vaccae* is able to tolerate and adapt to organic solvents. In order to accomplish this, alterations in the fatty acid composition of cells were assessed by lipid extraction followed by fatty acid analysis by gas chromatography; changes in the physicochemical properties of cell surface were analyzed regarding modifications in the cells' zeta potential; and fluorescence microscopy was used to analyze changes in cell morphology.

MIC in the presence of levofloxacin, teicoplanin, thioridazine and omeprazole was determined for cells previously adapted to high solvent concentrations and compared with those of non-adapted cells, in order to understand if there is cross-resistance between these two classes of compounds, and to study if efflux pumps are involved in acquired cross-resistance.

2. Materials and Methods

2.1 Microorganism

Mycobacterium vaccae ATCC 15483 was used in this study. It is deposited at the Institute for Bioengineering and Biosciences (iBB), Lisbon, Portugal, and stored at -80ºC. Periodically the cells are grown on agar plates and stored at 4ºC.

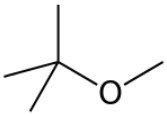
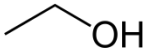
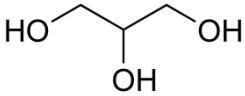
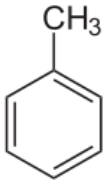
2.2 Chemicals

Culture media components were Mueller-Hinton (MH) broth from Sigma-Aldrich (Fluka Analytical) and Tween 80 from Merck-Schuchardt. MH was prepared by diluting 23 g of powder in 1 L of distilled water and adding 0.1% (v/v) of Tween 80. Mineral medium was prepared by adding 10 mL/L of 100x concentrated Phosphate Buffer and 10 mL/L of 100x concentrated Mineral Medium to 1 L of deionized water. The Mineral Medium solution was prepared by the addition of the following order of salts to 1 L of deionized water: 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 $MgCl_2.6H_2O$ (all from Sigma-Aldrich). 100x concentrated Phosphate Buffer was prepared by adding 55 g/L K_2HPO_4 and 85 g/L $NaH_2PO_4.H_2O$ (Sigma-Aldrich) to deionized water. The media were then autoclaved during 20 minutes at 121ºC, and stored at 4ºC.

The agar plates used to preserve the bacterial cells were prepared by adding 10 mL/L of 100x concentrated Mineral Medium, 10 mL/L of 100x concentrated Phosphate Buffer, 15 g/L of agar, 5 g/L of glucose and 3.5 g/L of yeast extract.

The solvents (Table 2) used in this work were ethanol (>99.9%) from Panreac, toluene (>99.5%) from Riedel-de Häen, MTBE (>99.5%) from Fluka Analytical, and a glycerol solution (86-89%) from Sigma-Aldrich.

Table 2 - Organic solvents used and information regarding its chemical structure, *log* P value (values retrieved from the literature) and zeta potential. The zeta potential of organic solvents was obtained as stated in section 2.5.

Organic solvent	*Chemical structure*	*Log P*	*Zeta potential (mV)*
MTBE		0.94	-6.10
Ethanol		-0.24	-8.29
Glycerol		-1.8	-16.2
Toluene		2.5	-21.7

The antibiotics used in this work were levofloxacin (LEVO) (Sigma-Aldrich; stock solution 1 mg/mL) and teicoplanin (TEICO) (Sigma-Aldrich; stock solution 1 mg/mL; 10 mg/mL). Efflux pump inhibitors (EPIs) were thioridazine (THIO) (Sigma-Aldrich; stock solution 1 mg/mL; 10 mg/mL) and omeprazole (OME) (Sigma-Aldrich; stock solution 4.5 mg/mL ethanol; 10 mg/mL DMSO (>99.5%, Merck KGaA)).

2.3 Growth Conditions

M. vaccae colonies were selected from agar plates and used as a pre-inoculum in 20 mL of Mueller-Hinton (MH) media supplemented with 0.1% Tween 80, in 100 mL Erlenmeyer flasks, and incubated in an Agitorb 200 (Aralab) at 30ºC and 200 rpm. The same procedure was used in the subsequent cell cultures derived from the pre-inoculum.

Solvent exposure: Once cells reached mid-exponential phase (OD 0.7-1.2) in MH medium, solvent was added at different concentrations to each cell culture. Growth rate (μ) was calculated based on incubation time with linearization and normalization of optical density (OD) measurements, using only exponential phase data. Each assay was done in duplicate.

Growth inhibition (%): $\frac{\mu 1,solv}{\mu 0,control} \times 100$

Carbon source: Mineral Medium was used to study if the cells were able to use the solvents as sole carbon and energy source, and supplemented with 0.1% Tween 80, in a total volume of 20 mL, in 100 mL Erlenmeyer flasks. Amounts of 0.25 and/or 1% (v/v) solvent were added to each Erlenmeyer, and a cell suspension of mid-exponential phase cells was added, to obtain an initial OD of 0.086. Growth was monitored by optical density measurements (at 600 nm) in a spectrophotometer U-2000 (Hitachi), and the cultures were incubated at 30ºC and 200 rpm. Assays were done in duplicate.

Adaptation: To conduct bacterial adaptation to ethanol and MTBE, 40 mL of MH media supplemented with 0.1% Tween 80 were used. When cells reached the mid-exponential phase, pulses of 1% (v/v) MTBE or 5% (v/v) ethanol were added. After the lag phase induced by the solvent was over, more pulses of solvent were added to the cultures once they reached exponential phase. Growth was monitored and maintained in the same conditions as the other experiments. Assays were done in duplicate.

2.4 Fatty acid composition

Mycobacterial cell suspensions were centrifuged at 10,000 rpm during 5 minutes in a µSpeedFuge SFA13K from Savant Technologies, and the pellet was washed twice with mili-Q water. The cell fatty acids were simultaneously extracted from the cell pellet and methylated to fatty acid methyl esters (FAMEs). FAMEs were prepared using the instant-FAME method from MIDI, Inc. and analyzed by gas chromatography on a 6890N gas chromatograph from Agilent Technologies, with a flame ionization detector and with an automatic injector 7683B equipped with a 25 m long Agilent J&W Ultra 2 capillary column from Agilent (Carla C C R de Carvalho et al., 2007). 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 saturation degree was calculated dividing the sum of saturated fatty acids by the sum of monounsaturated fatty acids.

2.5 Zeta potential

Cell suspensions (40 µL) were collected before and during solvent exposure, washed 3 times, and suspended in 2 mL of a 10 mM KNO_3 solution. The electrophoretic mobility of mycobacterial cells was determined in a Doppler electrophoretic light scattering analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd.) at 25ºC, using a clear disposable zeta cell. The zeta potential was determined using the electrophoretic mobility as an indirect measure of cell surface charge, according to the method of Helmholtz–von Smoluchowski (Hiemenz & Rajagopalan, 1986). The zeta potential of the organic solvents was also assessed for the solvents stated in 2.3, with a Glass “Dip” Cell (Malvern) for the

measurement of samples in aqueous and non-aqueous dispersants. To accomplish this, 1 mL of solvent was added to 2 mL of mili-Q water and centrifuged. In water-immiscible solvents where a second phase was formed, after centrifugation, 1 mL of the aqueous phase was retrieved and added to 2 mL of mili-Q water, being centrifuged again. An aliquot of 40 µL was collected and added to 2 mL KNO_3. Calculations were automatically performed using the Zetasizer software 7.10, from Malvern Instruments, Ltd.

2.6 Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) was determined for antibiotics and EPIs by the broth microdilution method in a 96-well microtitre plate (Sarstedt Inc.) according to CLSI guidelines (Woods et al., 2011) . Antibiotics and EPIs were serially diluted in two-fold steps, starting with different initial concentrations: LEVO (10 and 7.5 µg/mL), TEICO (100 and 75 µg/mL), THIO (149.3 and 125 µg/mL) and OME (500 and 400 µg/mL), in 150 µL of MH broth. Afterwards, 50 µL of a cell suspension collected in the exponential phase and diluted in MH broth to a 0.5 McFarland standard was added to each well. The experiments were performed in duplicate, both for solvent-adapted and non-adapted cells. The plate was then covered with a Breathe-Easy™ sealing membrane (Sigma-Aldrich), and kept without agitation at 30ºC. OD was measured after approximately 72h, at a wavelength of 600 nm, in a spectrophotometer SpectraMax® 340 PC from Molecular Devices.

2.7 Fluorescence Microscopy

2.7.1 Cell morphology

Cells were observed using a LIVE/DEAD® BacLight™ Bacterial Viability Kit from Molecular Probes which was added to the samples according to the instructions of the manufacturer. The microscope used was an Olympus CX40 equipped with an Olympus U-RFL-T burner and a U-MWB mirror cube unit (excitation filter: BP450-480; barrier filter: BA515). Images were collected with an Evolution™ MP 5.1 CCD color camera using the acquisition software Image-Pro Plus, both from Media Cybernetics (USA). One slide was made per sample and at least five images were taken from each slide.

2.7.2 Nile Red staining

In order to clarify the nature of the EPS observed in a few samples, Nile red was used to stain lipids. A stock solution of 1.3 mg/mL of Nile red (Molecular Probes, Life Technologies) in acetone was used and added to the sample to achieve a 1:100 dilution. This solution was added to the selected samples and kept in dark conditions for 5 min. The microscope used was an Olympus CX40 equipped with an

Olympus U-RFL-T burner and a U-MWG mirror cube unit (excitation filter: BP510-550; barrier filter: BA590). Images were collected with an Evolution™ MP 5.1 CCD color camera using the acquisition software Image-Pro Plus, both from Media Cybernetics. One slide was made per sample and at least five images were taken from each slide.

3. Results and Discussion

The degree of tolerance of *M. vaccae* towards toluene, ethanol, glycerol and MTBE was assessed, together with the analysis of the changes in the fatty acid composition of cells. To do this, cell suspensions were grown until mid-exponential phase in MH media and solvent was added. In order to understand if differences in the degree of tolerance were related with the use of solvents as carbon source, growth in mineral media was conducted. After these preliminary assays, two solvents were chosen to conduct cellular adaptation, and fatty acid composition, cell morphology and zeta potential were analyzed. To study the problematic of cross-resistance between solvents and antibiotics/EPIs, MIC determination was conducted in both solvent-adapted and non-adapted cells. Cells were then grown in ½ MIC of antibiotics and EPIs and fatty acid composition of cells was also investigated.

3.1 Solvent exposure

Cells were exposed to different concentrations of solvents in order to assess the degree of tolerance of *M. vaccae* towards these chemicals. Cells in mid-exponential phase (OD of 0.7-1.2) were exposed to different concentrations of toluene, ethanol, glycerol and MTBE. During the exposure time, fatty acid (FA) composition of cells was also examined.

3.1.1 Cells grown in the presence of toluene

Toluene and other aromatic hydrocarbons are known to be extremely toxic to living organisms due to its preferential partitioning to cell membranes, leading ultimately to membrane disruption and cell lysis (Sikkema et al., 1995). In fact, concentrations as low as 0.1% (v/v) are usually sufficient to kill most microorganisms (de Carvalho et al., 2007). In the present work, cells in exponential phase were exposed to 0.1, 0.5 and 1% toluene (Figure 7). Toluene was added approximately after 15 hours of growth when cells reached mid-exponential phase. It was possible to verify that in the cases where 0.5 and 1% toluene were added, there was an inhibition of the cellular growth.

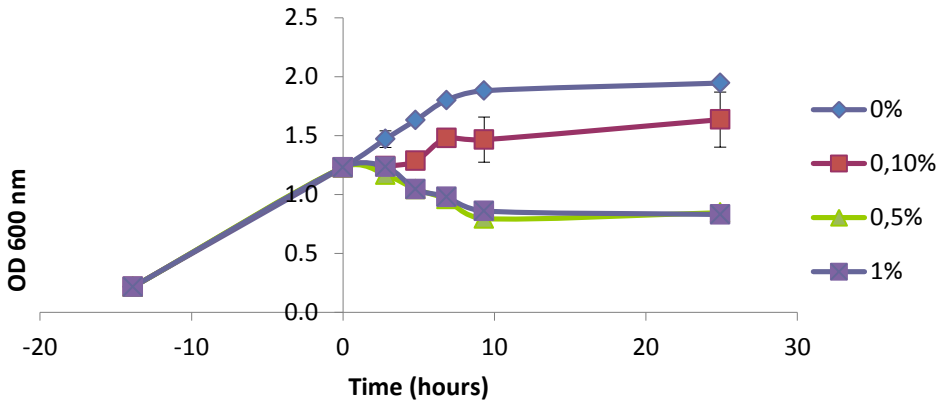


Figure 7 - Cells in the exponential phase exposed to 0, 0.1, 0.5 and 1% toluene.

In this work, *M. vaccae* seemed to exhibit a higher sensitivity to this compound than what was reported to other NTM mycobacteria. *M. austroafricanum* JOB-5 is able to catabolize and use toluene for cellular growth (Burback & Perry, 1993) and *M. cosmeticum* byf-4 is able to completely degrade toluene at an initial concentration of 100 mg/L within a time window of 36-42 hours (Zhang et al., 2013). In the present study, *M. vaccae* was unable to grow in concentrations higher than 0.1% (v/v) toluene and even after 45 hours of growth, the cells did not recover from the effect of this toxic compound (data not shown).

Fatty acid composition also changed in response to the different toluene concentrations tested. During control conditions (Figure 8 a)), the most common fatty acids present were 18:1 w9c (almost 60% of the total amount of FA in early hours), 16:0, 18:0, 12:0, 14:0 and 18:0 10-methyl. The most visible alterations under control conditions throughout time were a decrease in the amount of 18:1 w9c (58 to 48%) and 18:0 (10-5%), an increase in the content of 18:0-10 methyl (2 to 9%) and the appearance of 16:1 w6c FA after 7 hours of the beginning of the experiment, increasing from 8 to 11%.

When toluene was present (Figure 8 b, c and d), 16:1 w6c was not produced at all. In fact, the major differences observed between the different concentrations tested were associated with the production of 18:0 *iso* FA (2%). Also, there was an increase in 16:0 FA throughout time (between 16 and 21%), decreasing its amount in increasing toluene concentrations (only small variations from 16 to 17%), and a general decrease in 18:1 w9c (63-60%). In 0.5% there was a decrease in 18:0 throughout time (6.4% to 4.5%), whereas in higher concentrations, this value did not follow a specific trend with growth time. In 1% toluene, the amount of 16:0 FA was kept constant, increasing only from 7 to 11 hours of exposure (15.7 to 16.9%).

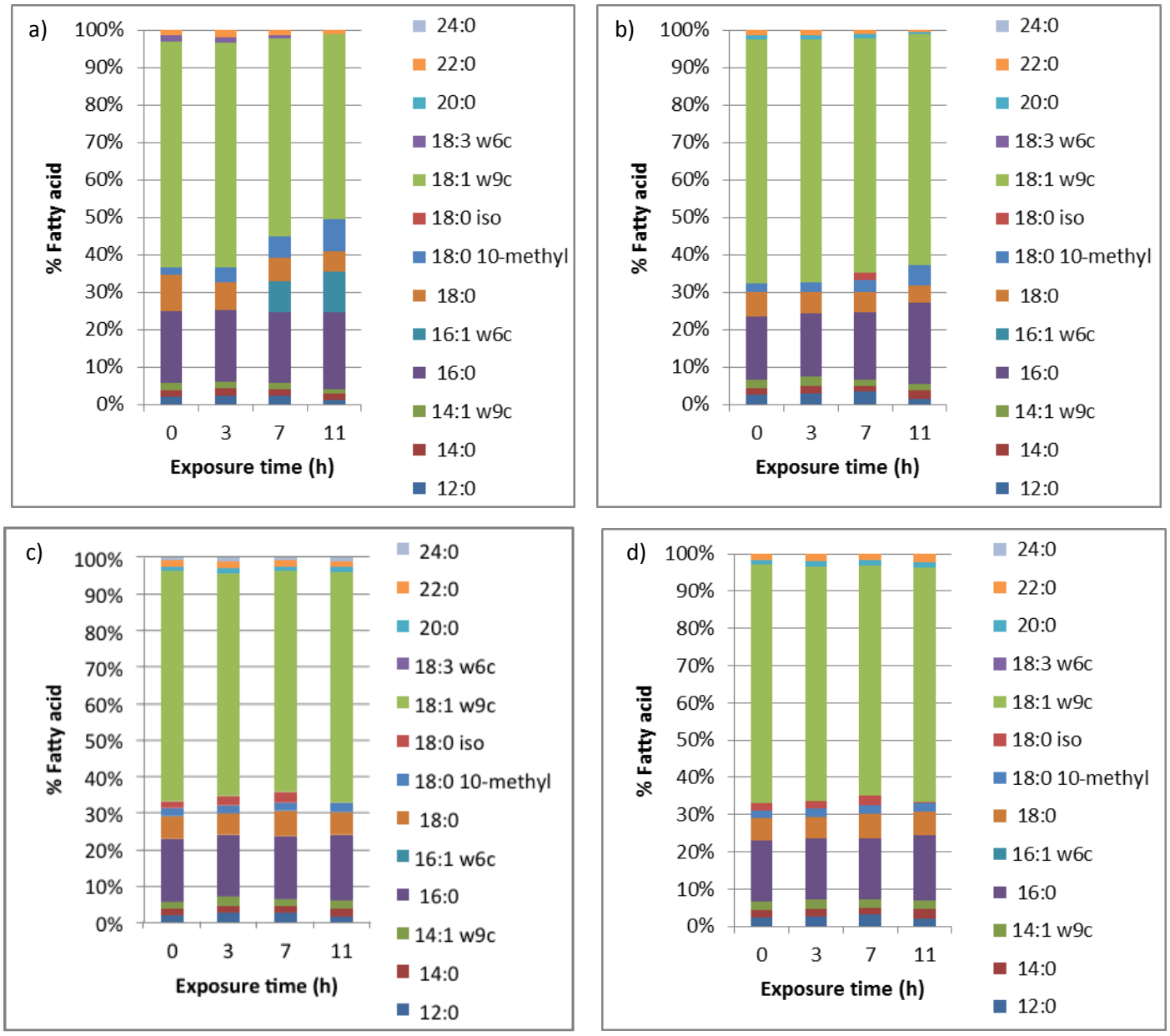


Figure 8 - Percentage of fatty acids present during toluene exposure, in a) 0, b) 0.1, c) 0.5 and d) 1%, at 0, 3, 7 and 11 hours of exposure.

In Figure 9, the proportion of polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), straight-chain saturated fatty acids (SSFA), branched-chain saturated fatty acids (BSFA), 10-methyl fatty acids and other fatty acids is depicted. There was an increase in the saturation degree of cells in the presence of toluene, which is in accordance to what was previously described for the closely-related *R. erythropolis* DCL14 (de Carvalho et al., 2007). There was an overall decrease in the content of MUFA (66 to 62%) and an increase of SSFA (30 to 32%) and 10-methyl fatty acids (2 to 5%) throughout time in cells exposed to 0.1% toluene. In 0.5% toluene, this trend was reverted at 11 hours of exposure, where there was a slight increase in MUFA (64 to 65%), BSFA (2 to 3%) and

SSFA (31 to 32%). In 1% toluene the content of SSFA increased throughout time (30 to 34%). When toluene is present at 0.5 and 1% (v/v), the amount of 10-methyl fatty acids remained constant throughout time (2%). This shows that when exposed to toluene, cells tended to increase the rigidity of the membranes in order to protect themselves from the action of toluene.

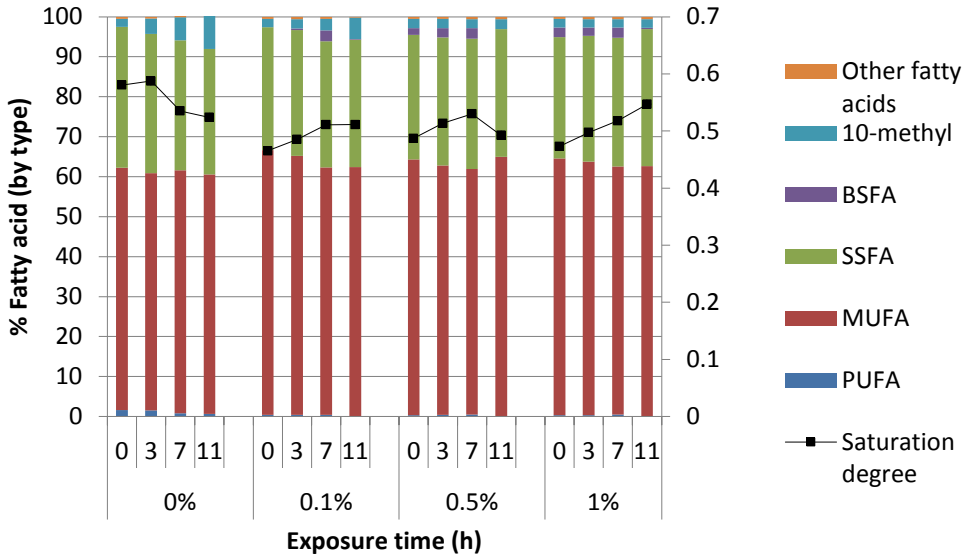


Figure 9 - Fatty acid composition of cells exposed to 0, 0.1, 0.5 and 1% toluene.

3.1.2 Cells grown in the presence of ethanol

Cells in mid-exponential phase were exposed to 1, 2.5 and 5% ethanol (Figure 10), which was supplemented to the cell culture at approximately 18 hours of growth. It was possible to see that according to the concentration used, the inhibition is better demarked. Nonetheless, even at 5% ethanol, the cells were able to grow, showing also signs of cell aggregation in all concentrations tested (observed at the naked-eye).

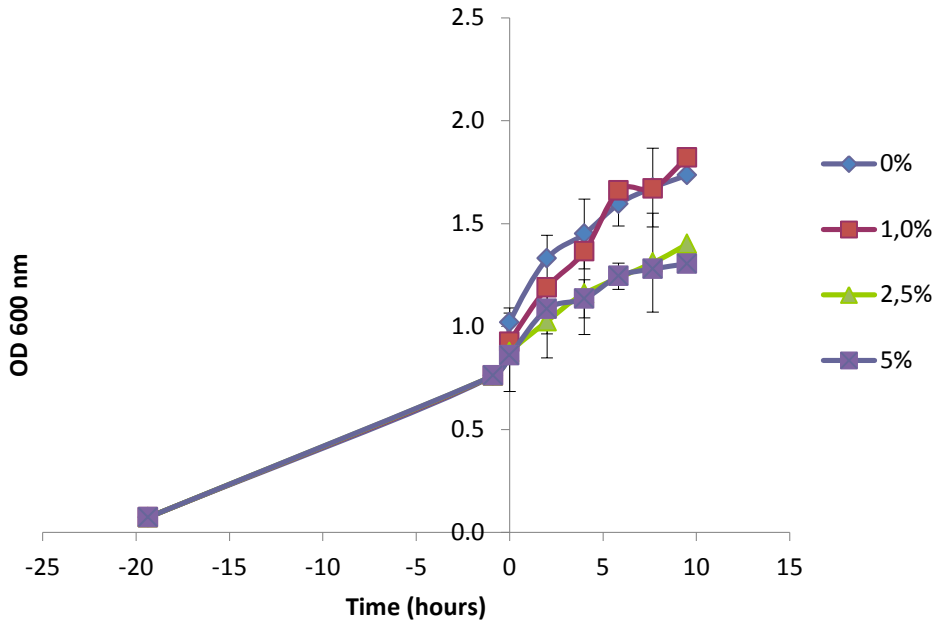


Figure 10 - Cells in the exponential phase exposed to 0, 1, 2.5 and 5% ethanol.

Alcohols interact directly with the lipid bilayer of the cells, leading to changes in the degree of freedom or fluidity of the cell membrane. It has been published that bacteria react differently to the presence of short-chain or long-chain alcohols. In the presence of short-chain alcohols such as ethanol, the saturation degree of cells decreases, whilst in the presence of long-chain alcohols it increases (de Carvalho et al., 2005; Heipieper, Weber, Sikkema, Keweloh, & de Bont, 1994; Ingram, 1976; Kabelitz, Santos, & Heipieper, 2003). This difference may be due to the fact that short-chain alcohols can only penetrate slightly into the hydrophobic center of the phospholipid bilayer, thus causing a swelling effect on the hydrophilic headgroups (Kabelitz et al., 2003). Therefore, the insertion of unsaturated fatty acids in order to counteract this effect seems a better adaptive response (Weber & De Bont, 1996). In *R. erythropolis* cells exposed to ethanol, a dose-dependent decrease in the membrane saturation degree was observed (de Carvalho et al., 2005).

Similar to the previous case, 16:1 w6c was produced after 7 hours of exposure and accounts for 10% of the total fatty acids present. Throughout time, 14:0, 16:0 and 18:0 10-methyl increased (only 2% in the former and 5% in the latter) and 12:0, 22:0, 24:0, 14:1 w9c, 18:0 (only after 11 hours of exposure) and 18:1 w9c decreased (1-2% variation, whilst 18:1 w8c was reduced from 63 to 49%).

In the presence of ethanol (Figure 11 b, c and d), 16:1 w6c FA was produced later, with the increase of solvent concentration. In 1% ethanol it was produced in a content similar to control conditions (approximately 8%), and, in 2.5% ethanol, it was only produced after 11 hours of exposure. In 5% ethanol, this FA was not produced at all. There was an overall decrease in 18:1 w9c throughout time, except in 5% ethanol, where this value increased from 0 to 7 hours of exposure (64 to 66%), only decreasing afterwards, until 11 hours (66 to 62%).

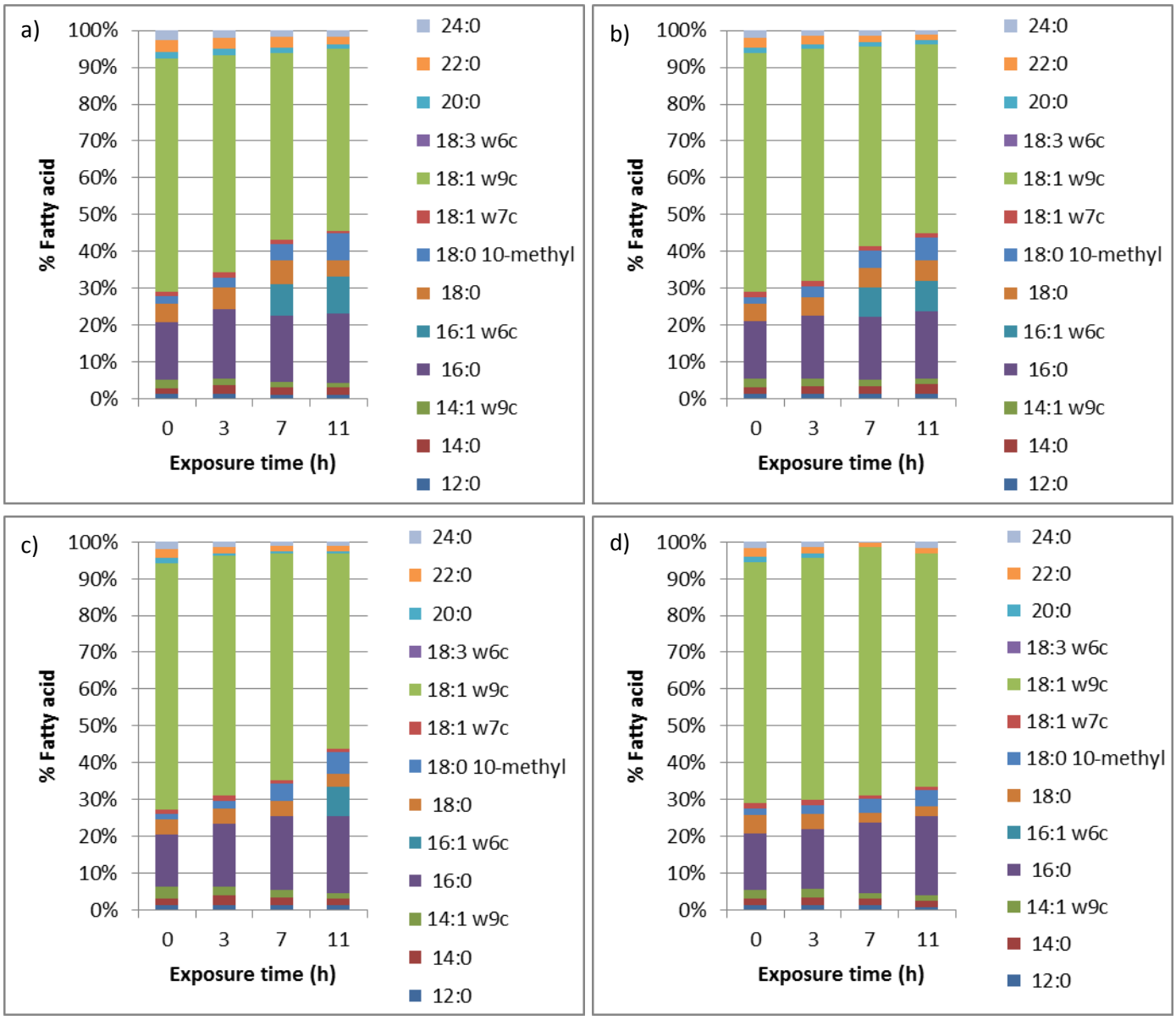


Figure 11 - Percentage of fatty acids present during ethanol exposure, in a) 0, b) 1, c) 2.5 and d) 5%, at 0, 3, 7 and 11 hours of exposure.

In this work, when cells were exposed to ethanol, the saturation degree of cells increased, a response contrary to what was expected based on the hypothesis stated before (Figure 12). This was also observed in other bacteria and in yeast, where ethanol acts by increasing the amount of saturated lipids, transmembrane lipids, sterols and hopanoids (Huffer, Clark, Ning, Blanch, & Clark, 2011). In 1 and 2.5% ethanol there was a 7% decrease in MUFA, and an increase in SSFA (1-2%) and 10-methyl FA (4-5%) throughout time. In 5% ethanol, MUFA and SSFA were kept constant in the first 3 hours of exposure (68 and 29%, respectively). After this time, MUFA decreased to 65% and SSFA increased to 31%. The amount of 10-methyl fatty acids increased throughout time in all concentrations, 2-6% in lower concentrations and 2 to 4% when 5% ethanol was present.

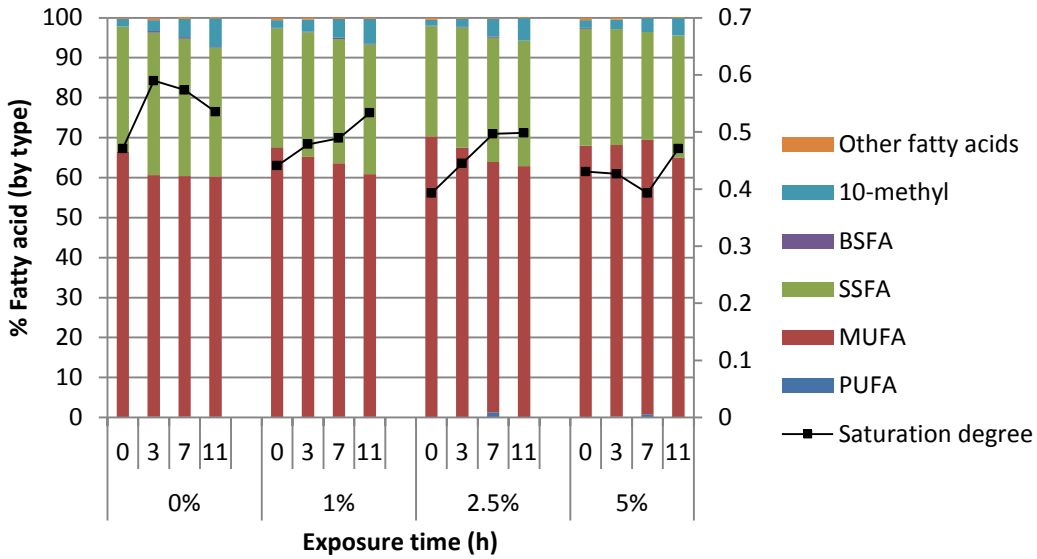


Figure 12 - Fatty acid composition of cells exposed to 0, 1, 2.5 and 5% ethanol.

3.1.3 Cells grown in the presence of glycerol

Glycerol was added to cell cultures in the exponential phase in amounts of 5, 10 and 10% (v/v). The higher the concentration of glycerol added to the cells, the higher the inhibition of growth when compared with control conditions (Figure 13).

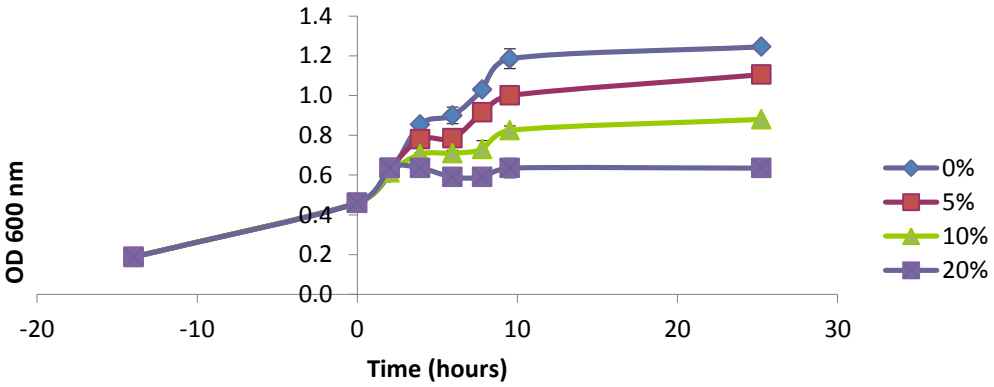


Figure 13 - Cells in the exponential phase exposed to 0, 5, 10 and 20% glycerol.

Contrarily to the previous cases, 16:1 w6c is always produced in the presence of glycerol, and the cells present a FA profile very similar to control conditions (Figure 14 b, c, d).

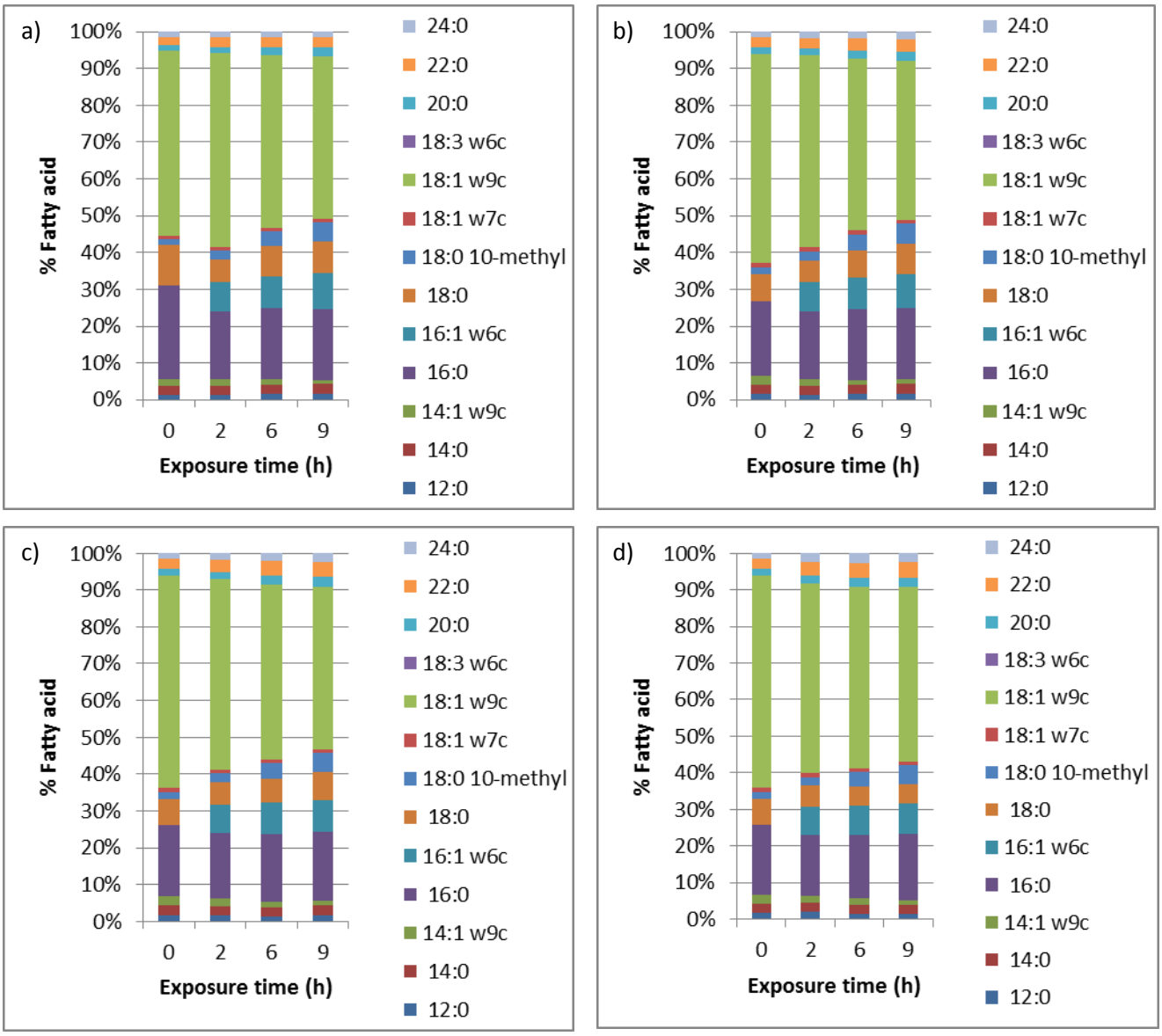


Figure 14 - Percentage of fatty acids present during glycerol exposure, in a) 0, b) 5, c) 10 and d) 20%, at 0, 2, 6 and 9 hours of exposure.

In a previous work with a *Mycobacterium* sp. MB-3683 using 0.5% glycerol as carbon source, 18:1 w9c, 16:0 and 18:0 10-methyl were the major fatty acids present after 144 hours of incubation (Borrego et al., 2000). Here, after 9 hours of exposure, the major fatty acids present are 18:1 w9c (42-47%), 16:0 (19-18%), 18:0 (8-5%) 16:1 w6c (9-8%) and 18:0 10-methyl (5%), showing that the higher the dose of glycerol added, the higher the amount of monounsaturated fatty acids and the lower the amount of saturated fatty acids.

After the addition of glycerol, mycobacterial cells increased the saturation degree of membrane fatty acids (Figure 15). Nonetheless, at early hours of exposure (2 hours), there was a decrease in the saturation degree of cells in all concentrations tested. This is in accordance to the increase in the content of MUFA at an exposure time of 2 hours (2%), which tended to decrease throughout time, together with an increase in the content of SSFA (8%) and 10-methyl fatty acids (2 to 5%) after two hours of exposure to the new conditions.

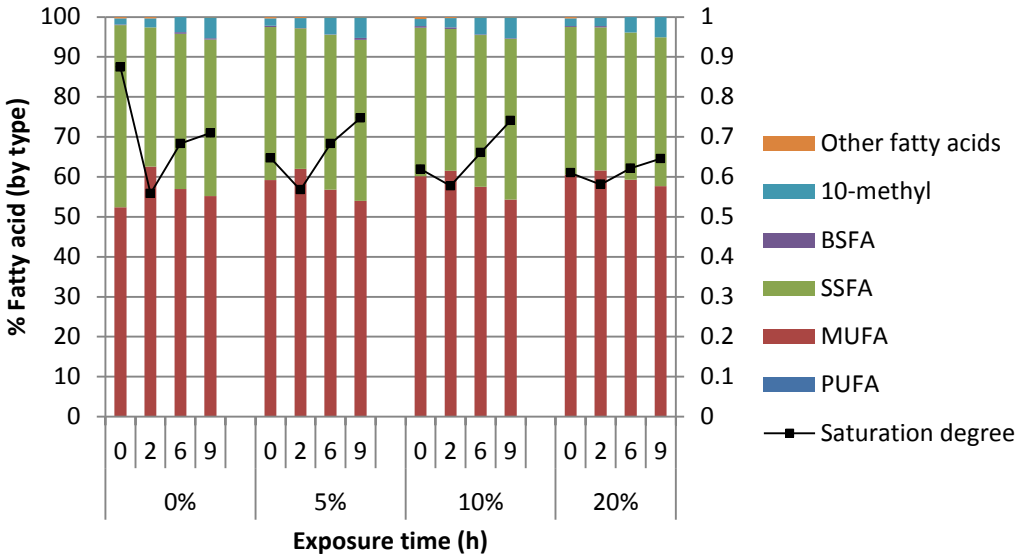


Figure 15 - Fatty acid composition of cells exposed to 0, 5, 10 and 20% glycerol.

3.1.4 Cells grown in the presence of MTBE

Cells were grown until reaching the exponential phase, in which different concentrations of MTBE were added to the cell cultures. Higher concentrations of solvent evidenced higher inhibitory effects, when comparing with the control, where no solvent was added (Figure 16). Several reports mention that mycobacteria can grow in MTBE or even mineralize it (Ferreira et al., 2006; Johnson, Smith, Reilly, & Hyman, 2004). *M. austroafricanum* JOB5 was shown to oxidize the primary metabolite of MTBE, called TBA, to several by-products, after growth on a wide range of n-alkanes, isoalkanes and BTEX compounds (House & Hyman, 2010).

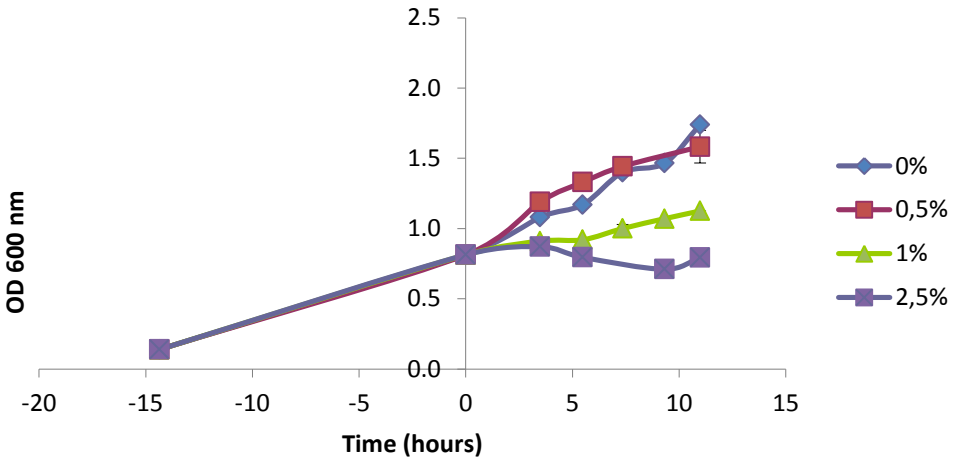


Figure 16 - Cells in the exponential phase exposed to 0, 0.5, 1 and 2.5% MTBE.

Under control conditions, there was an increase in the content of 14:0 (1 to 2%), 16:0 (11 to 18%), 22:0 (1 to 2%), 16:1 w6c (8 to 9% between 7 and 11 hours of exposure), 18:0 (3 to 7%) and 18:0 10-methyl FA (1 to 6%), and a decrease in 18:1 w9c (from 71% to 48%), and 14:1 w9c FA (4 to 1%) throughout time (Figure 17 a)). When MTBE was present (Figure 17 b, c, d)) at a concentration of 0.5%, the FA profile was very similar to control conditions. When cells were exposed to this concentration of solvent, 16:1 w6c FA was produced after 7 hours of exposure, same as in the case of control. In 1% MTBE, this FA appeared only after 11 hours of exposure, and in 2.5% MTBE it was not detected at all.

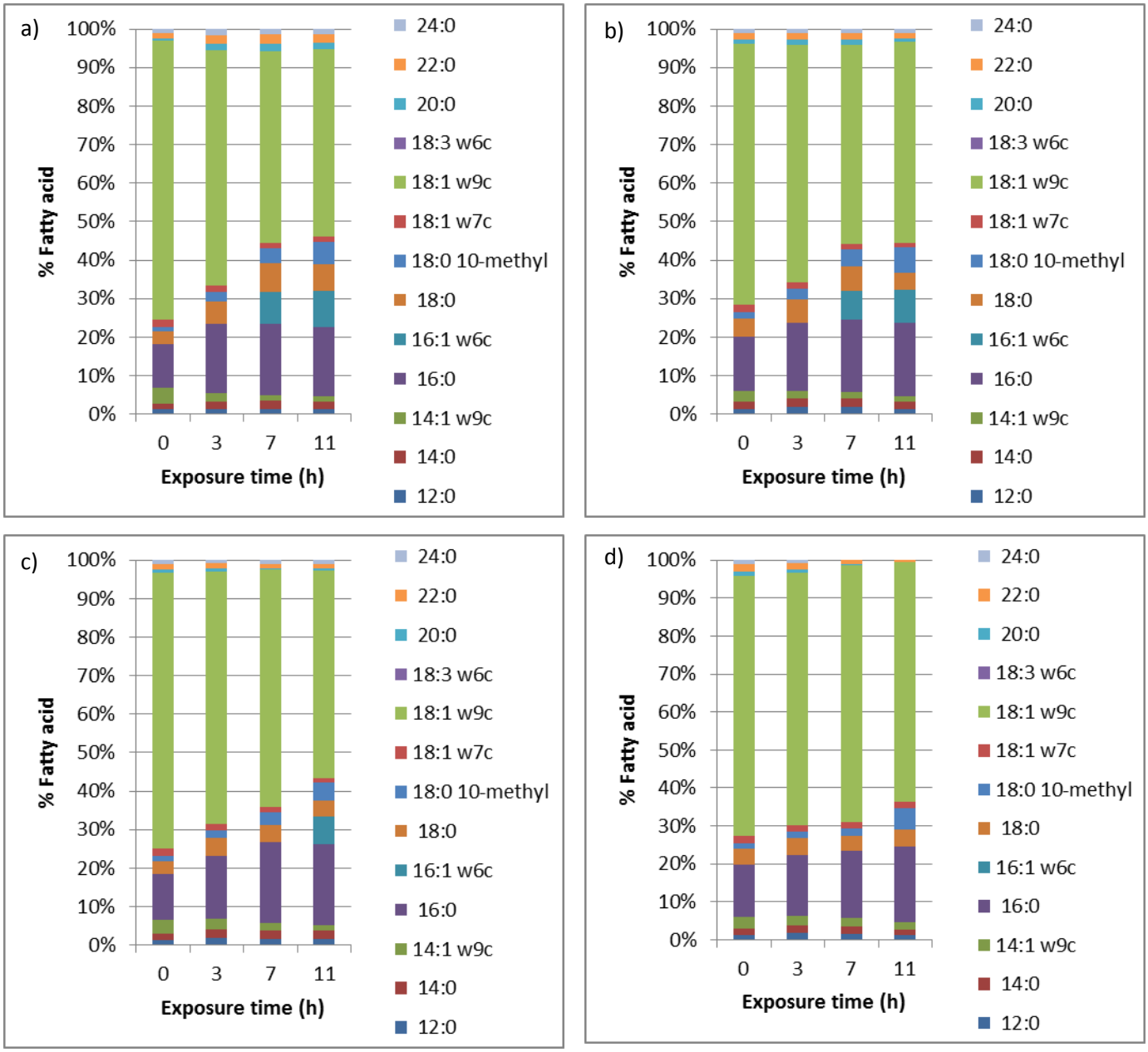


Figure 17 – Percentage of fatty acids present during MTBE exposure, in a) 0, b) 0.5, c) 1 and d) 2.5%, at time 0, 3, 7 and 11 hours.

The addition of MTBE to the cell cultures led to an immediate increase in the saturation degree of cells in 0.5 and 1% MTBE (Figure 18). In 0.5% MTBE, there was a decrease in the content of MUFA (71 to 62) and an increase of SSFA (26 to 31%). In 1% MTBE, the decrease in MUFA (76 to 63%) and the increase in SSFA (21 to 32%) was maintained throughout all the experimental time. In 2.5% MTBE, the same behavior was observed.

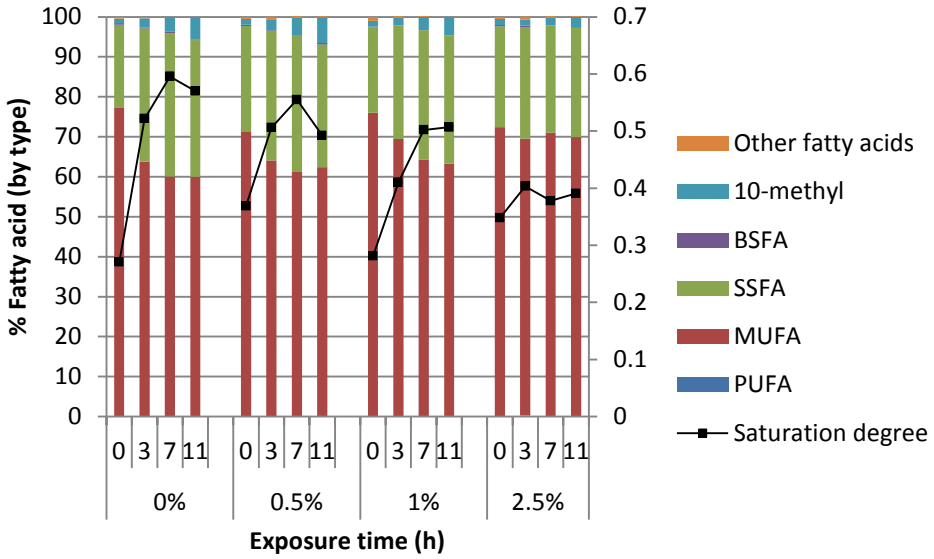


Figure 18 - Fatty acid composition of cells exposed to 0, 0.5, 1 and 2.5% MTBE.

By summarizing all the information regarding the alterations in the FA composition of cells in all solvent concentrations at the different experimental time-points, the disparities were evident. After 2-3 hours of solvent exposure, all conditions resembled a similar trend in the amount and type of FA produced, except in the presence of glycerol (Figure 19). In this case, 16:1 w6c FA was 8% of the total FA after 2 hours of exposure, exhibiting a similar trend as for control conditions, where no glycerol was present. It was also possible to verify that there was an increase in the saturation degree of cells with the increase of the amount of glycerol (0.56 to 0.58). The opposite was observed when toluene, ethanol and MTBE were present, since there was a decrease in the saturation degree of cells with the increase of the concentration of solvent tested.

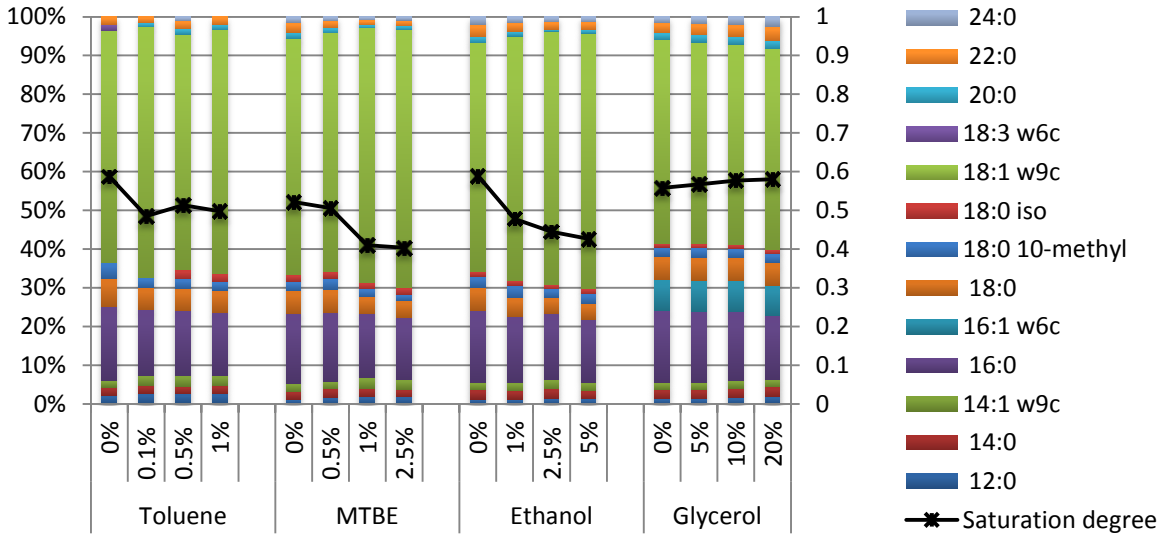


Figure 19 - Fatty acid composition of cells in all solvent concentrations tested, after 2-3 hours of exposure.

After 6-7 hours of solvent exposure, 16:1 w6c FA was still the major difference amongst the different conditions (Figure 20). This FA was present in the control of each experience (0% solvent), accounting for 8% of the total FA. It was also produced when 0.5% MTBE and 1% ethanol were present (7-8%) and again in all the cases where glycerol was added. It is clear that the presence of higher concentrations of MTBE and ethanol had an effect in cells that led to the inhibition of this FA, and that even at the lowest concentrations of toluene this FA was not produced. After this time, the saturation degree of cells decreased with increasing concentrations of solvent, contrarily to what happened in the previous time-point analyzed. As before, the saturation degree of cells also decreases with increasing concentrations of MTBE and ethanol. In the case of toluene, the saturation degree of cells was kept constant.

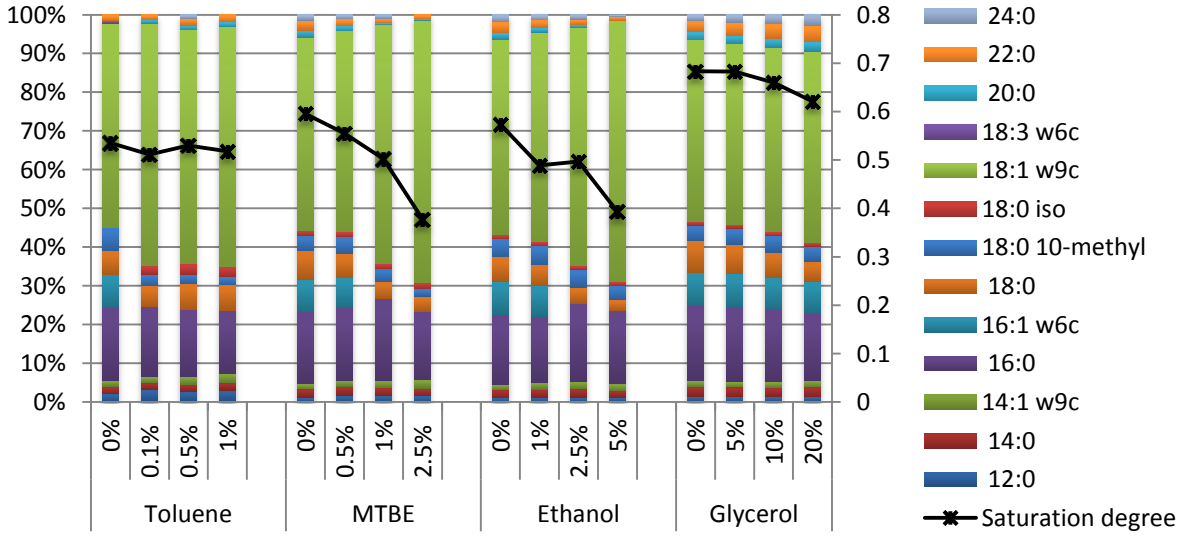


Figure 20 - Fatty acid composition of cells in all solvent concentrations tested, after 6-7 hours of exposure.

After 9-11 hours of solvent exposure, 16:1 w6c was produced in 1% MTBE and 2.5% ethanol (Figure 21). After this time, there was still no production of this FA in all toluene concentrations tested, neither in higher concentrations of MTBE and ethanol. This shows that the effect exerted in cells was similar, being the absence of 16:1 w6c a possible direct indicator of the solvent's toxicity. In higher concentrations of solvent, the growth rate of cells is lower. Combining all data gathered so far, there seems to be a relation between the production of this FA and the cellular capacity to overcome cell toxicity and grow. The saturation degree of cells decreased when MTBE and ethanol were present, with increasing concentrations of solvent. When glycerol and toluene were present, the saturation degree decreased in the presence of 20% glycerol and increased in the presence of 1% toluene.

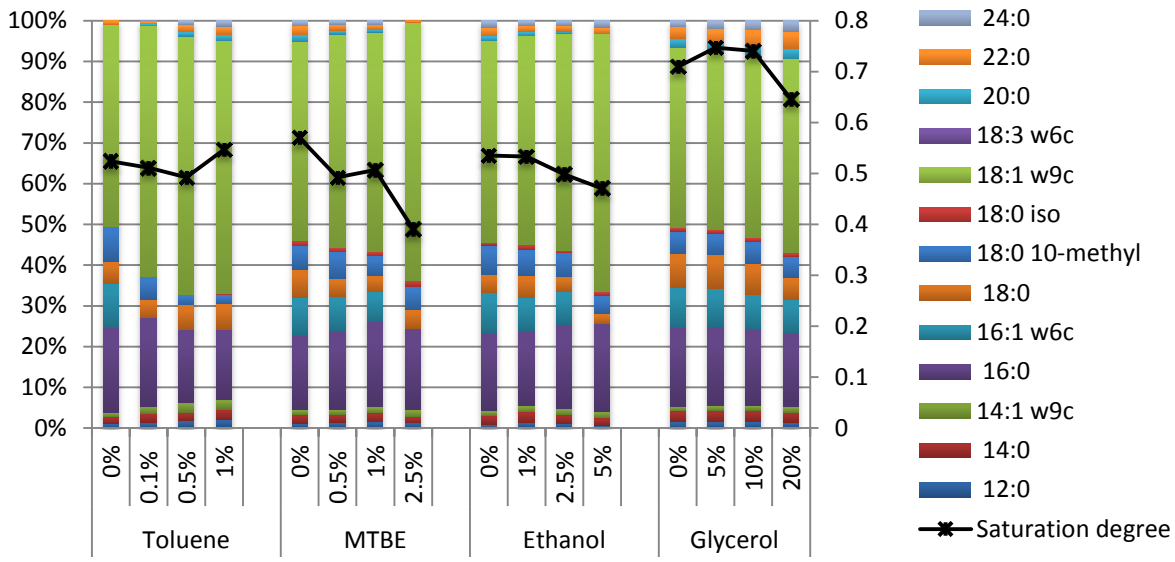


Figure 21 - Fatty acid composition of cells in all solvent concentrations tested, after 9-11 hours of exposure.

3.1.5 Growth inhibition

Growth inhibition was calculated as a function of the growth rate of cells exposed to solvents in comparison with non-exposed cells. When cells were supplemented with solvents, there was an immediate inhibition of the growth rate with the increase of the concentration, with exception for 1% ethanol, where the growth rate was even higher than control conditions (Figure 22 a)). Nonetheless, ethanol and glycerol were tolerated by the cells in higher concentrations than toluene or MTBE. In 0.5% toluene and 2.5% MTBE (Figure 22 b)), the growth was 0, meaning that even at low concentrations the effects of these compounds are extremely toxic and cells were unable to survive. In this work, solvent toxicity could not be predicted based on its *log P* value. Cells were able to tolerate glycerol better than ethanol (*log P* values of -1.8 and -0.24, respectively) and MTBE better than toluene (*log P* values of 0.94 and 2.5, respectively). Also, cells can have a better profile in the presence of organic solvents that can be mineralized or used as carbon source.

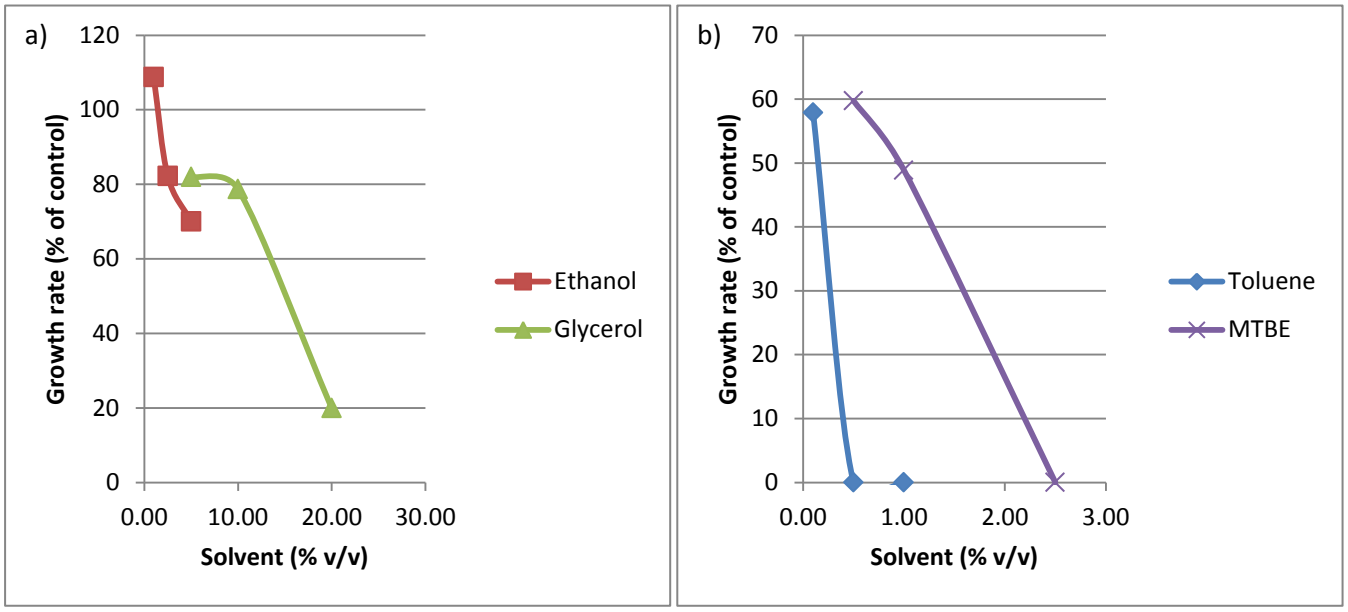


Figure 22 – Growth inhibition after solvent supplementation relative to control conditions, in a) ethanol and glycerol and b) toluene and MTBE.

3.2 Solvents as carbon source

In order to assess if *M. vaccae* could use the organic solvents tested as single carbon and energy sources, Mineral medium was used, supplemented with 0.25 or 1% (v/v) ethanol, glycerol, toluene and MTBE, and the cell cultures were maintained for approximately 80 hours (Figure 23).

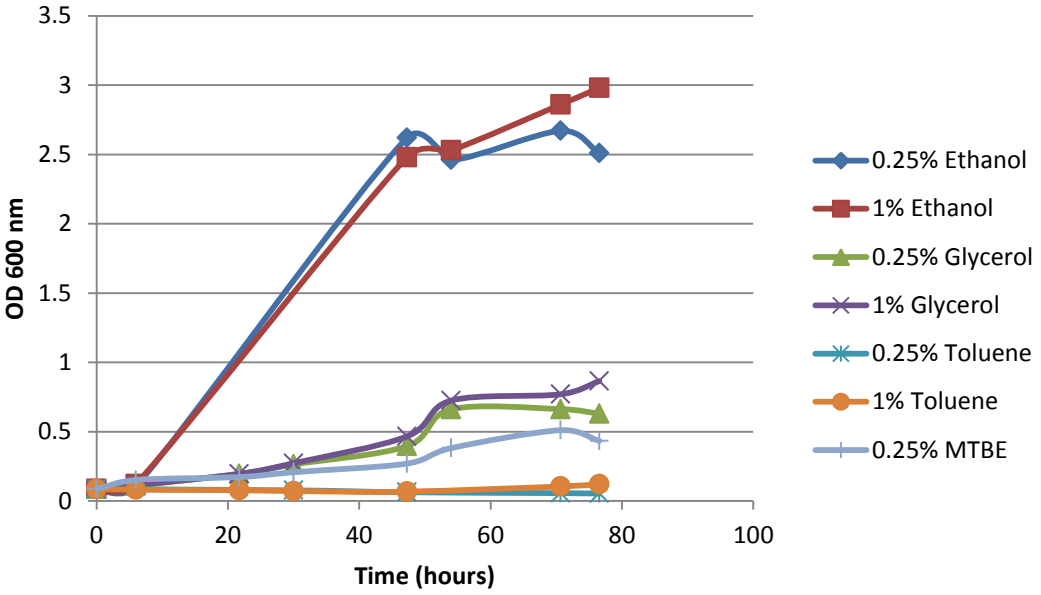


Figure 23 - Growth in mineral media, using organic solvents as sole carbon source.

Starting with an initial OD of 0.086, mycobacterial cells were able to grow perfectly well either in 0.25 or 1% ethanol, reaching ODs of 2.5-3. These cells also seem to use glycerol and MTBE as sole carbon source, but to a less extent than ethanol. As for glycerol, after 80 hours, maximum ODs of 0.7 were obtained. In 0.25% MTBE, this value was lower, being only 0.5. Cells were unable to use toluene as sole carbon and energy source.

In the presence of ethanol as sole carbon source, *R. erythropolis* DCL14 was able to grow in concentrations up to 20% (de Carvalho & da Fonseca, 2005). Glycerol is considered to be the favored carbon source of *M. tuberculosis* and *M. bovis* BCG under laboratorial conditions. The wild type *M. bovis*, however, is unable to use glycerol as sole carbon and energy source due to a nucleotide polymorphism in the pykA gene (Beste et al., 2009; Keating et al., 2005). Variability can be also found in species that can use MTBE. *M. vaccae* JOB5 (currently *M. austroafricanum* JOB5) does not grow on MTBE when it is supplied as a sole carbon and energy source (Johnson et al., 2004), but more recently discovered strains *M. austroafricanum* IFP 2012 and *M. austroafricanum* IFP 2015 were able to grow on MTBE as a sole carbon and energy source and to degrade it with different catabolic efficiencies (Ferreira et al., 2006). *M. cosmeticum* byf-4 was also reported to use toluene as sole carbon source (Zhang et al., 2013).

When 0.25% toluene was present as sole carbon source, even after 60 hours, *R. erythropolis* was unable to grow, presenting thus a very large lag phase. Nonetheless, when concentrations around 0.125% were added, the cells were able to grow (de Carvalho & da Fonseca, 2005). Later, this strain evidenced a remarkable ability to adapt and perfectly grow in the present of concentrations up to 50% (v/v) toluene (de Carvalho et al., 2007). The concentrations selected for toluene supplementation might thus have been too high or the experimental time too insufficient.

3.3 Cellular adaptation to increased concentrations of ethanol and MTBE

Adaptation of cell populations to stresses is based on the contribution of the most tolerant individuals, due to differences in genetic and physiological diversity. Cell adaptation can be ascribed to: i) enzyme-mediated tolerance (where the toxic compounds are degraded into non-toxic products) (Ma, Hager, Howell, Phibbs, & Hassett, 1998); ii) genetic adaptation; iii) physiological state; iv) adaptation of the membrane composition (Pieper & Reineke, 2000); v) efflux pumps that extrude the toxic compound (Kieboom, Dennis, Zylstra, & de Bont, 1998). Slow-growers or cells in the stationary phase acquire general tolerance to stress, and the slowing of growth is seen as a signal of tolerance acquisition (Booth, 2002) and as an inducer of separate but overlapping pathways which permit the acquisition of tolerance to several stresses (de Carvalho, Poretti, & Da Fonseca, 2005).

In order to test if solvent-adapted cells present an improved tolerance towards antibiotics and EPIs when compared with non-adapted cells, cellular adaptation was carried out throughout 70 hours. Since mycobacteria exhibited a good profile when grown in 5% ethanol and 1% MTBE, these concentrations were selected to conduct the experiment. Cells were grown until the exponential phase and solvent was added. When the lag phase induced by the addition of the first pulse of solvent seemed to be over, subsequent pulses were administered to the culture.

Adaptation to ethanol was carried out by adding three pulses of 5% (v/v) of ethanol (Figure 24), totalizing a final content of 15% (v/v) ethanol. Cells in the presence of this solvent only grew until a maximum OD of 1.6 and, after the second pulse, formed visible aggregates. After each pulse, there was an immediate decrease in the OD of the culture, followed by a subsequent recovery.

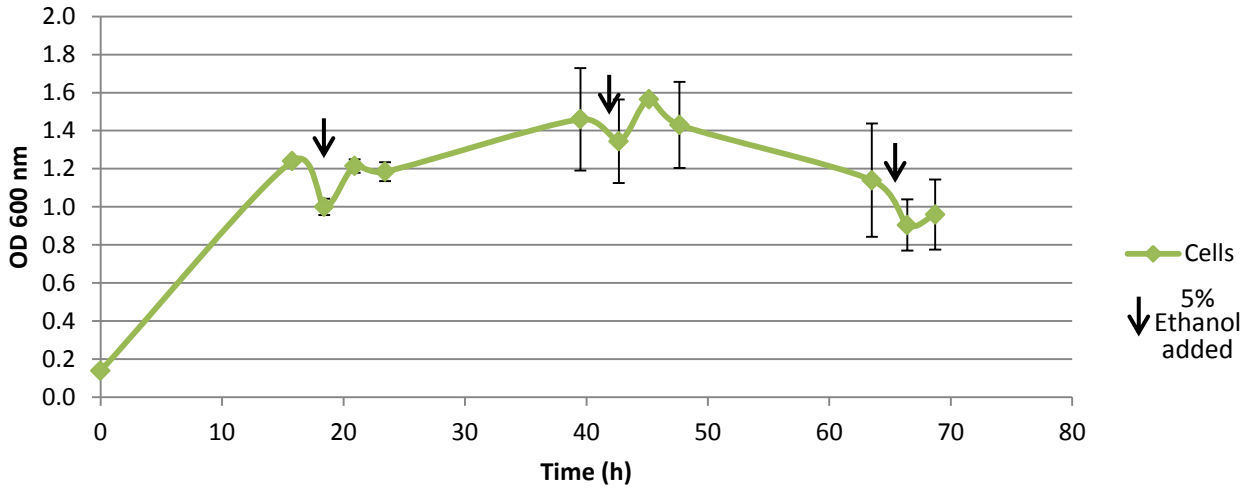


Figure 24 - Growth curve obtained by the measurement of the OD of the culture, subjected to three pulses of ethanol, in the time points indicated by the arrows.

In the case of MTBE-adapted cells, an OD of approximately 7 was achieved (Figure 25). Four pulses of 1% (v/v) MTBE were added to the cultures, without the formation of visible aggregates. Nonetheless, in this case, the color of the culture was somehow blurry, same as during MTBE exposure in 3.1.4. Interestingly, after the first pulse, the cell culture exhibited a better growth profile in the subsequent two pulses.

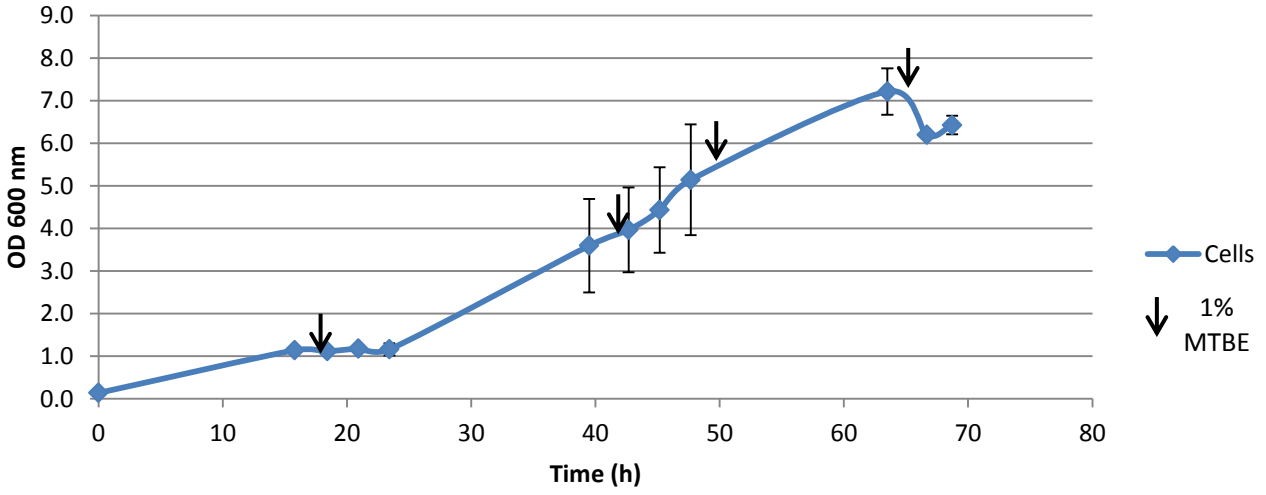


Figure 25 - Growth curve obtained by the measurement of the OD of the culture, subjected to four pulses of MTBE, in the time points indicated by the arrows.

The zeta potential of cells during adaptation was measured at different times of the experiment. At the beginning of the experiment (OD =0.139), the zeta potential of *M. vaccae* cells was approximately -44.975 mV. When cells were at the exponential phase, the zeta potential became more negative (-49.3 mV). The increase of negative charges when cells entered the exponential phase was already described for *M. smegmatis* mc2155, as a result of a change in the metabolic stage of cells, since they are actively dividing (Ayala-Torres et al., 2013). When cells reached the exponential phase, the first pulse of solvent was added. The zeta potential of cells changed accordingly to the type of solvent used throughout the adaptation time. Ethanol-adapted cells were more negatively charged when compared with MTBE-adapted cells (Figure 26). The influence of the growth substrate on the surface charge of cells was already observed (de Carvalho et al., 2009; Wick et al., 2002). In *R. erythropolis* DCL14, the alkane chain-length also had an influence in the cell polarity (de Carvalho et al., 2009).
The difference observed in zeta potential can be related with the effect of the solvents in cells and prior adjustment of the physicochemical properties of the cell surface in order to overcome the presence of the toxic compounds. Membrane proteins contribute to the total net charge of the cell surface and can alter the zeta potential through variation in their amounts and through the establishment of different intermolecular interactions (Tokumasu, Ostera, Amaratunga et al., 2012) The presence of cellular aggregation is a demonstration of the high hydrophobicity caused by the presence of ethanol, thus being in accordance with a more negative zeta potential.

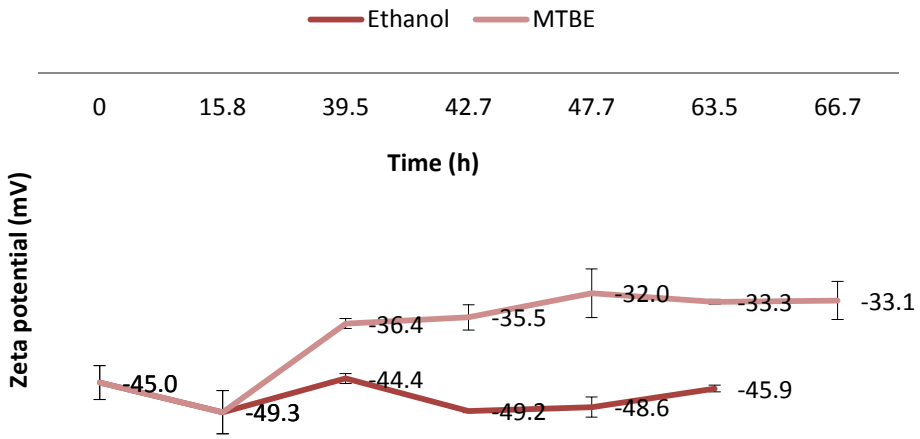


Figure 26 - Zeta potential (mV) of cells during adaptation to ethanol and MTBE.

The morphology of *M. vaccae* cells was analyzed using fluorescence microscopy, enabling the visualization of both viable (green) and non-viable (red) cells. The samples were collected after 45 hours of growth, in cells subjected to two pulses of ethanol and three pulses of MTBE.
In the case of ethanol-grown cells, two types of cellular features were distinct: the presence of large aggregates of cells and shrinked cells (Figure 27). The amount of red-stained cells was reduced, being an indicator of a high percentage of viable cells in the sample. Previous reports stated that the

establishment of cell clusters, biofilms or microcolonies provides a better protection against membrane-active compounds. When a population is organized as a microcolony, there is an increase of the total extracellular space inside the colony, together with a reduction of the colony surface area to volume (Heipieper, Keweloh, & Rehm, 1991). Also, the cells inside the colony are protected from the toxic effects of the solvent.

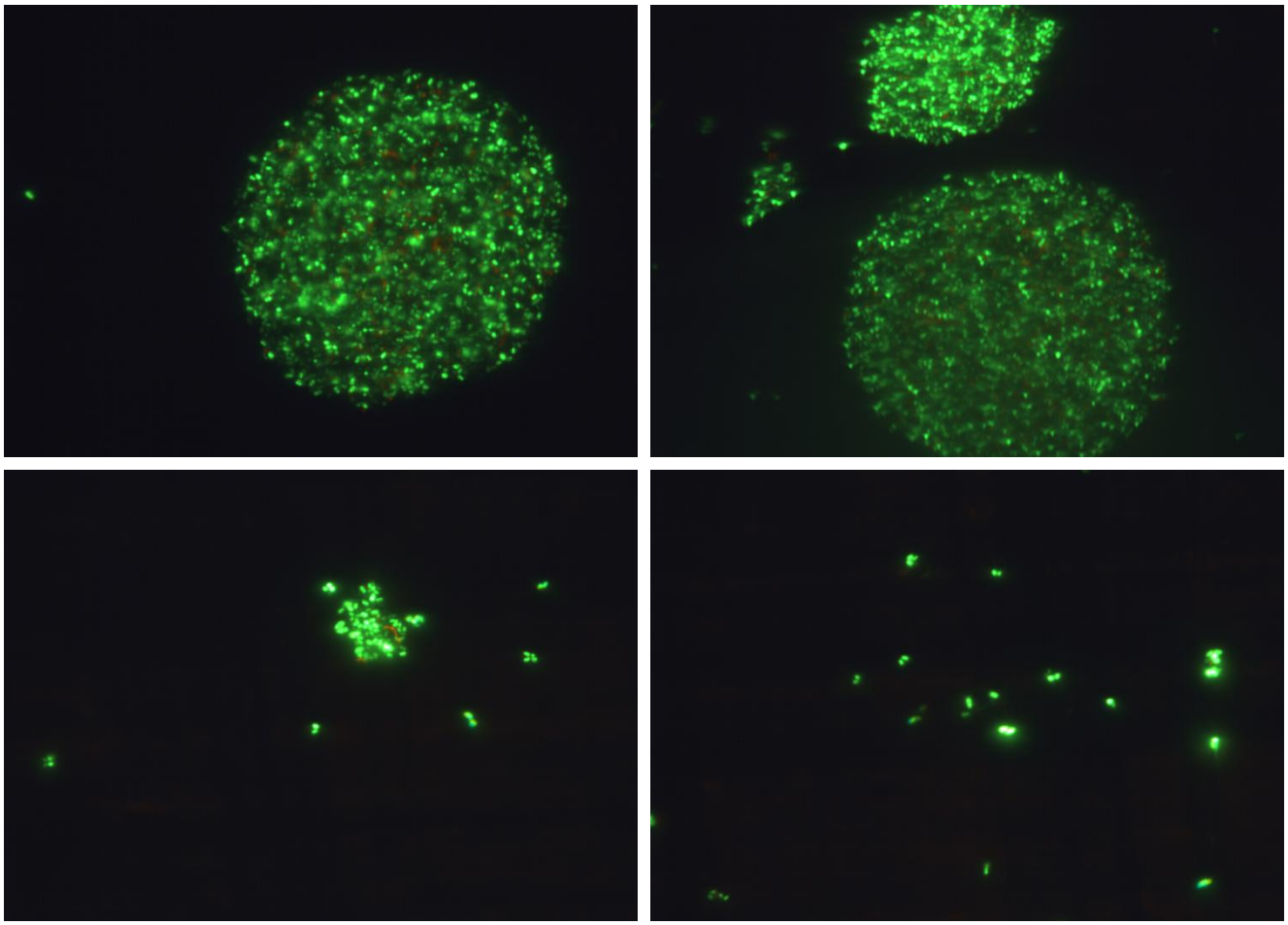


Figure 27 – Cells grown in the presence of ethanol after 45 hours of growth. In the first two images it is possible to see the large aggregates and in the images below, the presence of shrinked single cells. Almost all cells remain viable after 30 hours of exposure to ethanol. Magnification 1500x.

As for MTBE-grown cells, both cellular aggregation and elongated rods were observed. Changes in cell morphology in the presence of toxic compounds were previously reported both in Gram-negative bacteria, where an overall increase in cell size was observed, and in Gram-positive bacteria, where filamentous growth, increase in cell volume and production of extracellular capsules was perceived (Murínová & Dercová, 2014; Nielsen, Kadavy, Rajagopal, Drijber, & Nickerson, 2005; Zahir et al., 2006). An increase of cell size leads to a decrease of the ratio between surface and volume of cells, thus reducing the relative surface and consequently diminishing the available area for the interaction with organic solvents. This may be also implied as an improvement of the function of efflux pumps, since these pumps end up being more effective when the membrane surface is reduced (Neumann et al., 2005). In this case, there was a high amount of red-stained cells, showing that a part of the population lost the viability after an incubation period of 30 hours in MTBE (Figure 28).

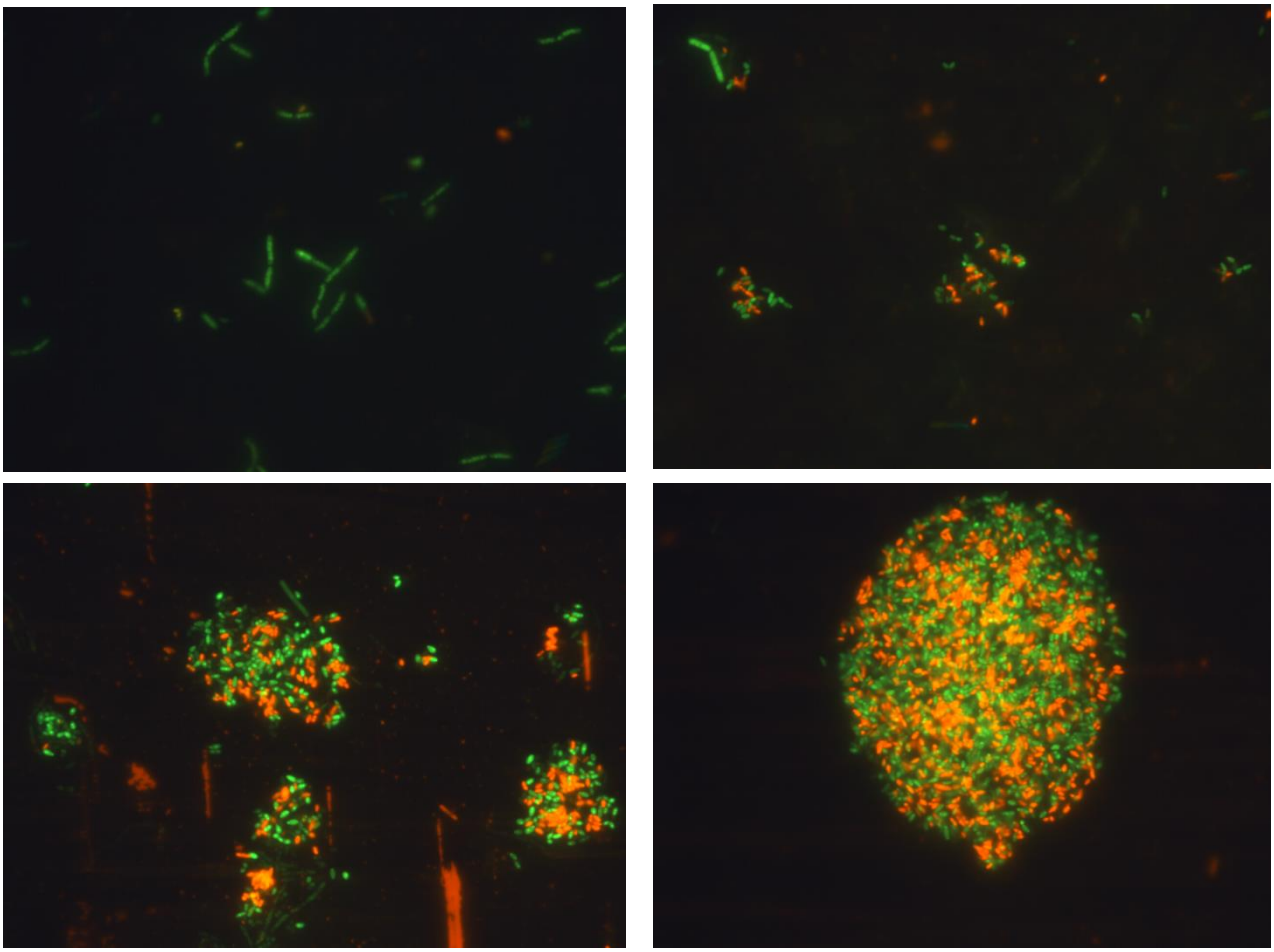


Figure 28 - Cells grown in the presence of MTBE after 45 hours of growth. In the first two images it is possible to see rod-shapped cells and in the images below, the presence of aggregates. A significant part of the cell population lost viability after 30 hours of exposure to MTBE. Magnification 1500x.

The adaptation of a cell population towards a toxic compound is linked with the ability of bacterial cells and its progeny to remain viable under conditions that would inhibit or kill other members of the population (de Carvalho et al., 2005).

Adaptation to ethanol and MTBE led to significant changes in the fatty acid composition of cells. During cellular growth until exponential phase (0 to 16 hours), a small decrease in the saturation degree of cells was verified, indicating a fluidization that might have been associated with cellular growth. At the beginning (t0), the most common fatty acids present were 18:1 w9c (67%), 16:0 (13%), 18:0 (8%), 12:0 (4%) and 14:0, 14:1 w9c and 18:1 w7c (3% of each) (Figure 29). When MTBE was added, *iso* and *anteiso* FA were produced. There was an increase in 13:0 *iso* (1 to 3%), 14:0 *iso* (0.7 to 1.4%), 15:0 *iso* (7 to 13%), 15:0 *anteiso* (5 to 6% until 48 hours, where it kept decreasing until 4.7%), 16:0 *iso* (2 to 4%), 17:0 *anteiso* (3 to 4% until 64 hours and decreases after 69 hours),17:0 *iso* (7 to 12%) and 19:0 *iso* (it is kept constant at 1% through time and decreases after 69 hours). Throughout time, there was an increase in 14:0 (1 to 2%), 16:0 (12 to 13%) and a decrease in 18:1 w9c (43 to 29%), 16:1 w6c (5 to 3%) and 18:0 10-methyl (3 to 2%).

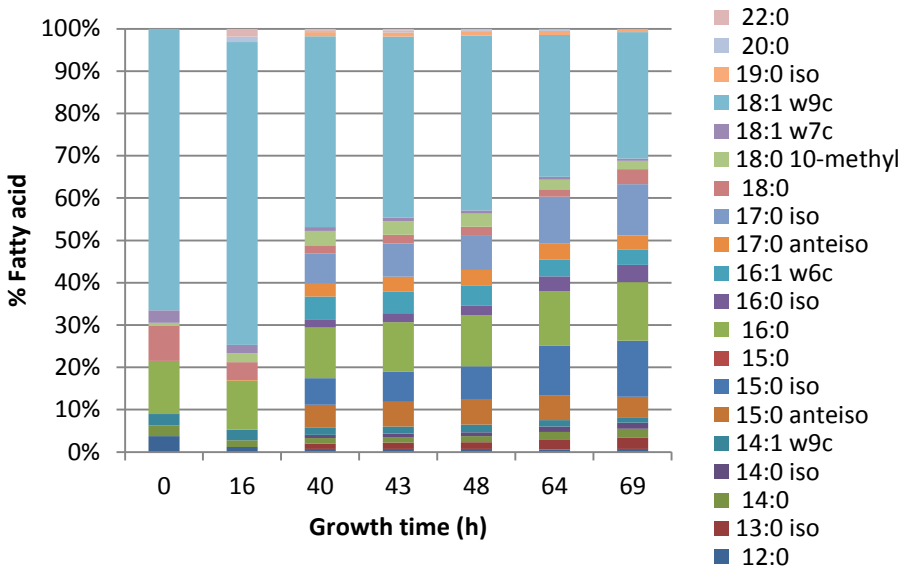


Figure 29 - Percentage of fatty acids present during adaptation to MTBE.

From 0 to 16 hours, there was a decrease in the saturation degree, indicating a fluidization of the cell membrane (Figure 30). When MTBE was added, there was a decrease in MUFA throughout time, until after 69 hours of adaptation this value was only 35%. The amount of SSFA decreased from 16 to 40 hours (23-17%), but increased later throughout the rest of the adaptation time. After 40 hours of adaptation, BSFA represented 27% of the total FA content, increasing until it achieved a value of 42% after 69 hours. The amount of 10-methyl FA increased until 43 hours (1 to 3%) and decreased throughout time, until it was only 1% after 69 hours of growth.

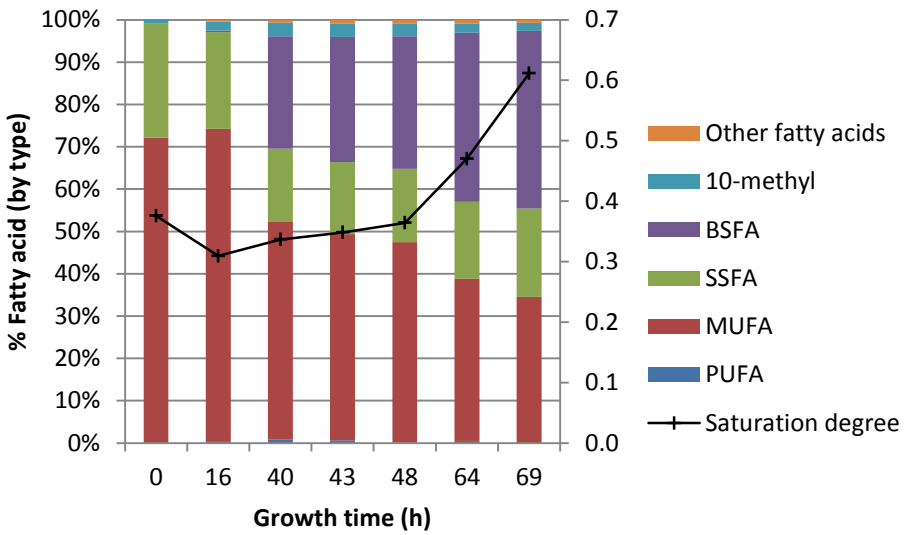


Figure 30 –Fatty acid composition of cells during adaptation to MTBE.

An increase in the amount of *iso*-branched fatty acids decreases the fluidity or flexibility of the cellular membrane (Carla C C R de Carvalho et al., 2007). In this case, cells decreased membrane fluidity and increased the saturation degree, leading to an increase in the rigidity of the cell membrane as a way of protection against the toxic effect of MTBE. This behavior is in accordance with what was previously observed in section 3.1, during MTBE-exposure. Nonetheless, the previous experiment was not prolonged enough to detect if the production of BSFA appeared with the increase of the exposure time or if it was a result of the addition of several pulses of solvent.

During ethanol adaptation, between 0 and 16 hours of incubation, there was a decrease in 12:0 (4 to 1%), 18:0 (6.5 to 4%), 18:1 w7c (3 to 2%) and 18:1 w9c (70 to 69%), an increase in 18:0-10 methyl (1 to 2%) and 22:0 (2%) and 20:0 (1%) FA appears (Figure 31). After 16 hours the first pulse was applied. The second pulse was applied after 40 hours of incubation, and the most notorious alterations at the level of the FA were a decrease in the amount of 16:0 (24.5 to 23%), 18:0 (3 to 2%) and an increase in 16:1 w6c (8.7 to 9%), 14:1 w9c (0.5 to 0.9%) and 18:1 w9c (49 to 52%). After the last addition of solvent, the most prominent changes were at the level of 18:1 w9c (58 to 51%), 16:1 w6c (10 to 8%), 16:0 (20 to 21%), 18:0 (2 to 3%) and 18:0 10-methyl (3 to 6%).

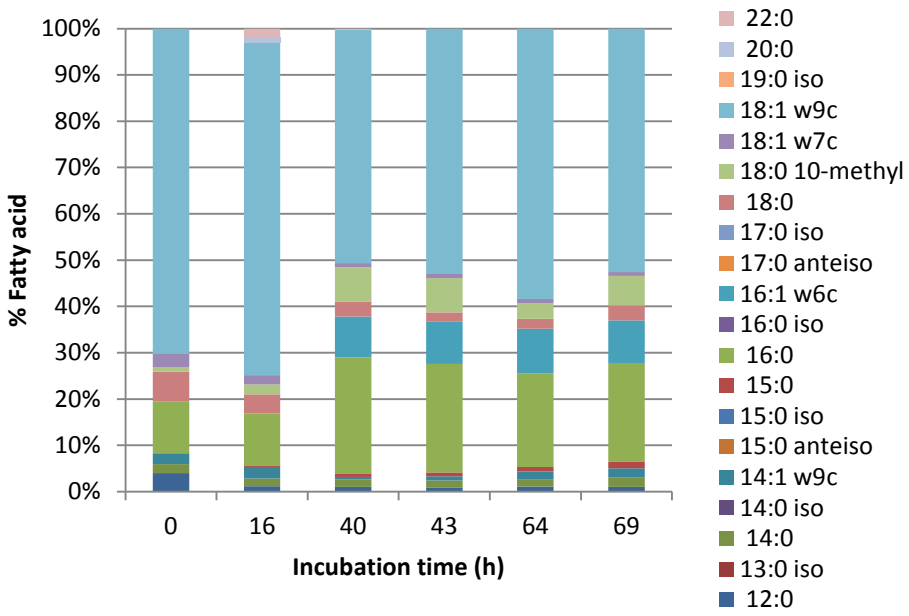


Figure 31 - Percentage of fatty acids present during adaptation to ethanol.

As for cells grown in ethanol, it was possible to observe an increase in the saturation degree of cells after the first pulse of ethanol. Three pulses of solvent were added to the culture, at 16, 40 and 64 hours. After the first pulse of solvent, the amount of MUFA decreased from 74 to 60% and SSFA and 10-methyl increased from 23 to 32% and 2 to 7%, respectively (Figure 32). After the second pulse it was possible to see a decrease in the saturation degree of cells, maintained until the 64 hours, where the third pulse was applied. The fatty acid composition after the second pulse was dominated by an increase in the content of MUFA, from 60 to 63%, and a decrease in SSFA from 32 to 29%. After the third pulse, there was again an increase in the saturation degree of cells. Whilst in the case of MTBE-grown cells, BSFA appeared after 40 hours of exposure, in the case of ethanol-grown cells, only 1% of BSFA was detected at 16 and 69 hours. There was again an increase in the content of SSFA, from 27 to 29% and a decrease in MUFA, from 70 to 63%. The fatty acid profile at 43 and 69 hours was quite similar, corresponding to time points almost immediately after the addition of ethanol pulses.

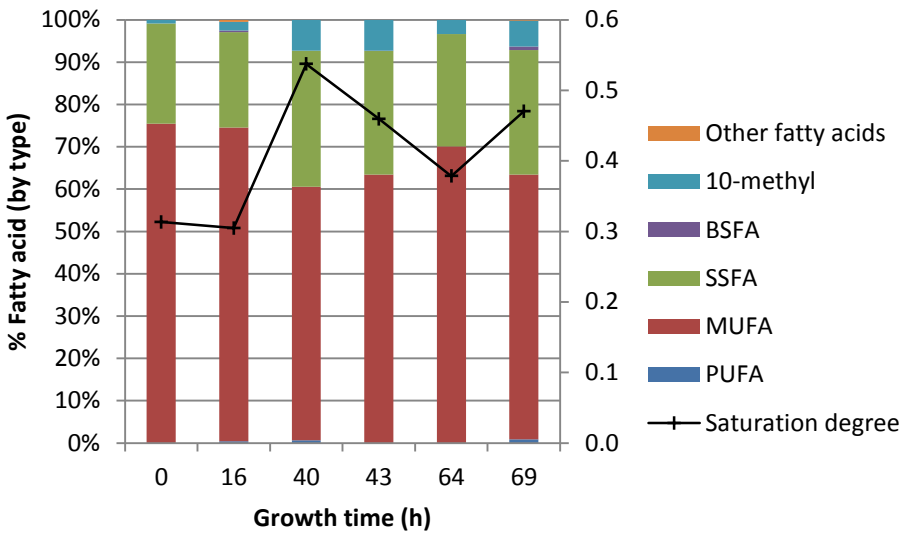


Figure 32 - Fatty acid composition of cells during adaptation to ethanol.

3.4 Minimum inhibitory concentration (MIC) determination

Due to the increasing concern towards infections caused by NTM, in 2003 the CLSI has established criteria for antimicrobial susceptibility testing (AST) of NTM and has defined recommendations for the AST method. The document was essentially based on clinical data, organism population distribution and expert experience in NTM (Brown-Elliott, Nash, & Wallace, 2012). The standard method for rapidly-growing mycobacteria is broth microdilution. The criteria are designed for a lot of NTM species, but, due to the lack of data available, some species are not included in the guidelines (Brown-Elliott et al., 2012). This is the case of *M.vaccae*, which was not mentioned in the document. Since this species is a rapidly-growing mycobacteria such as *M. fortuitum*, the same criteria were applied.

Teicoplanin and levofloxacin were used as model antibiotics and thioridazine and omeprazole as model EPIs. Levofloxacin is known to possess activity against non-turberculous mycobacteria such as *M. abcessus* and *M. fortuitum* (Brown-Elliott et al., 2012) and *M. tuberculosis* (Rastogi, Goh, Bryskier, & Devallois, 1996) and teicoplanin is a glycopeptide agent used in clinical cases, being seen as a useful antibiotic in tuberculosis drug development (Kieser et al., 2015). Teicoplanin acts as an inhibitor of bacterial wall synthesis and levofloxacin inhibits both DNA gyrase and topoisomerase IV, both involved in DNA replication.

MIC determination is very important as an assessment of the inhibitory effects of efflux pumps. Omeprazole is a protein pump inhibitor (H^+-K^+-ATPase) and it is known to inhibit the NorA pump of Gram-positive bacteria, being currently applied in clinical uses (Tegos et al., 2013). Thioridazine belongs to the group of piperidine compounds and it acts by blocking calcium channels, thus inhibiting

ABC type efflux pumps. It was reported as being able to inhibit the efflux of ethidium bromide both in *M. smegmatis* and *M. avium* complex (Rodrigues et al., 2008).

In order to characterize the susceptibility of *M. vaccae* towards these compounds, the MICs were determined, and cellular assays were carried out in ½ of the MIC. MIC determination was also conducted in both MTBE and ethanol-adapted cells (Table 3, Table 4).

Table 3 - MICs determined for TEICO and LEVO regarding *M. vaccae* ATCC 15483.

	Non-adapted Cells		*MTBE-adapted cells*		*Ethanol-adapted cells*	
Antibiotics	**MIC** (µg/mL)	½ MIC (µg/mL)	**MIC** (µg/mL)	½ MIC (µg/mL)	**MIC** (µg/mL)	½ MIC (µg/mL)
Teicoplanin	**>100**	50	**50**	25	**25**	12.5
Levofloxacin	**0.6**	0.3	**0.6**	0.3	**0.04**	0.02

Non-adapted cells presented higher tolerance levels to teicoplanin than MTBE and ethanol-adapted cells. In the case of levofloxacin, the MIC of non-adapted cells was the same as for MTBE-adapted cells, but this value decreased 15-fold when compared with ethanol-adapted cells.

A previous report presented a MIC value of ≤ 0.25 µg/mL for levofloxacin, using the same *M. vaccae* ATCC 15483 (de Moura et al., 2012). Non-tuberculous mycobacteria display a wide range of susceptibility towards levofloxacin. The *in vitro* activity of this drug against 17 species of atypical mycobacteria was characterized, and MIC values ranging between 0.125-8 µg/mL were obtained (Rastogi et al., 1996). According to the CLSI guidelines, *M. vaccae* is susceptible to levofloxacin. As for teicoplanin, several *M. tuberculosis* strains exhibited MIC values >64 µg/mL (Arain, Goldstein, Scotti, & Resconi, 1994). Other sources claim that teicoplanin and other glycopeptides are not effective against mycobacteria, fungi or gram-negative rods (Craig & Stitzel, 2004). This drug is not contemplated in the CLSI guidelines, since it is not approved for use in the United States, and so no information regarding susceptibility testing was available.

This results are coherent to what was previously described for other mycobacteria and point to the conclusion that adaptation to these solvents did not lead to increased resistance towards the antibiotics tested, and ethanol-adapted cells were more susceptible to these compounds than MTBE-adapted cells.

Table 4 – MICs determined for THIO and OME regarding *M. vaccae* ATCC 15483.

	Non-adapted Cells		*MTBE-adapted cells*		*Ethanol-adapted cells*	
EPIs	**MIC** (µg/mL)	½ MIC (µg/mL)	**MIC** (µg/mL)	½ MIC (µg/mL)	**MIC** (µg/mL)	½ MIC (µg/mL)
Thioridazine	**18.7**	9.35	**74.6**	37.3	**74.6**	37.3
Omeprazole	**250**	125	**500**	250	**500**	250

In the case of EPIs, MTBE and ethanol-adapted cells presented higher MIC values when compared with non-adapted cells, and both MTBE and ethanol-adapted cells presented equal MIC values towards these compounds. Different *M. tuberculosis* strains had MIC values of 15 µg/mL for thioridazine (Viveiros, 2015). Atypical mycobacteria such as *M. avium* ATCC 25291 and *M. smegmatis* mc^2155 presented the same MIC value for thioridazine, 20 µg/mL (Rodrigues et al., 2008). As for omeprazole, no study concerning Mycobacterium was found. Nonetheless, the closely-related *R. erythropolis* DCL14 presented a MIC value of 320 µg/mL towards this compound (Vencá, 2012). The results obtained were consistent with the ones described on literature.

This shows that the use of EPIs in the treatment of mycobacterial infections can be hindered by the previous contact of environmental mycobacteria with organic solvents, due to adaptation mechanisms.

After this screening, cells were grown in the presence of ½ MIC of each antibiotic and EPIs, enabling the maintenance of cell viability. This assay was carried out both in cells adapted to solvents and non-adapted cells, growth was monitored by OD measurements and fatty acid composition analysis was carried out. The same or approximate initial OD was used in both assays.

3.4.1 Non-adapted cells grown in ½ MIC of antibiotics and EPIS

Non-adapted cells were grown in 1% MTBE plus ½ MIC of antibiotics and EPIs and 5% ethanol plus ½ MIC of antibiotics and EPIs. Cells were initially cultured with solvent and 50 µg/mL of TEICO, 0.3 µg/mL of LEVO, 9.35 µg/mL of THIO and 125 µg/mL of OME were added independently. This assay was carried out approximately during 80 hours.

Under control conditions, where only 1% MTBE was present, cells experienced a slower growth rate in the first 30 hours of exposure, reaching only an OD of 1.88. After this time, an exponential growth was observed between 30 and 54 hours of growth. This prolonged lag phase may be associated with the toxicity of MTBE. The cells achieved a final OD of 7.2 under control conditions, 2.04 with LEVO, 3.57 with TEICO, 3.07 with THIO and 3.21 with OME (Figure 33). In the presence of MTBE and OME, a small increase in the OD was detected until 22 hours of growth, but this value decreased and was

maintained constant, only to start increasing after 70 hours of growth. In cells grown with MTBE and THIO and MTBE and LEVO, lag phase lasted until approximately 54 hours of growth.

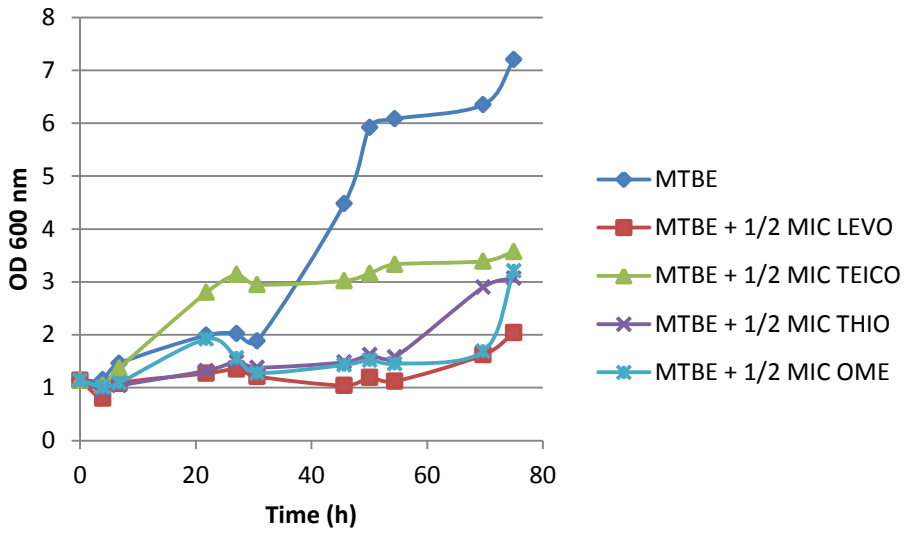


Figure 33 – Non-adapted cells grown in the presence of 1% MTBE and 1/2 MIC of antibiotics and EPIs.

Cells in the exponential phase were grown in 5% ethanol, and ½ MIC of antibiotics and EPIs (Figure 34). Starting with an initial OD of 1.114, final ODs of 1.29, 4.77, 0.71 and 2.53 were obtained in cells exposed to 5% solvent plus LEVO, TEICO, THIO and OME, respectively. Under control conditions, an OD of 2.96 was obtained. Cells were able to achieve higher OD when subjected to the synergistic toxicity of both ethanol and TEICO, than ethanol alone. When exposed to ethanol and TEICO, the lag phase lasted until 30 hours, where cells started growing in an exponential manner. Almost no growth occurred in cells exposed to ethanol and LEVO, and a decrease in the OD of cells was immediately verified in cells exposed to ethanol and THIO. After 70 hours of growth, in cells exposed to ethanol and OME the OD of cells started increasing.

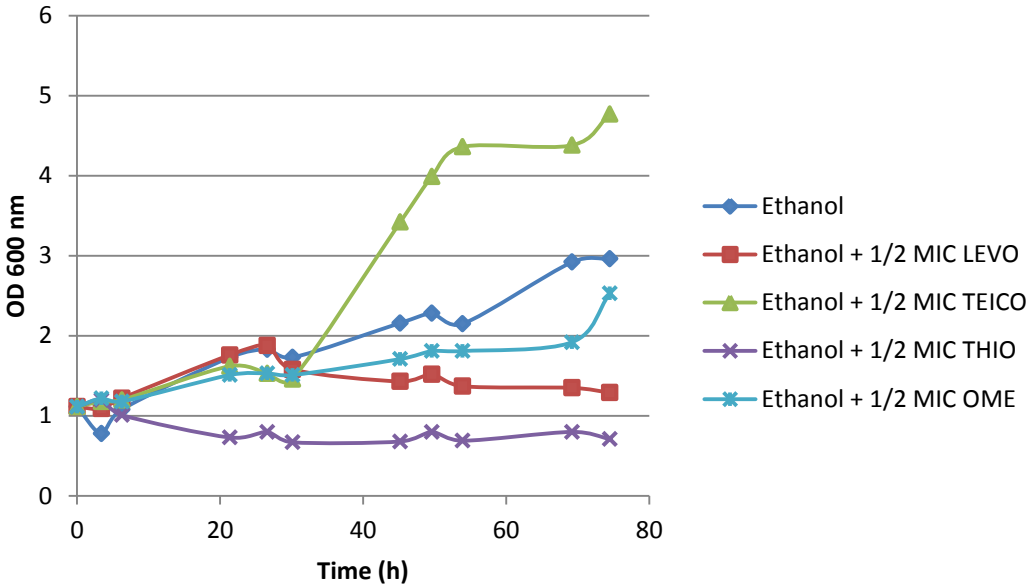


Figure 34 – Non-adapted cells grown in the presence of 5% ethanol and 1/2 MIC of antibiotics and EPIs.

The fatty acid composition of cells was analyzed throughout several time points of the experimental time. Before adding MTBE, at time 0, the major fatty acids present are 18:1 w9c (41%), 18:0 10-methyl (17%), 16:0 (15%) and 16:1 w6c (9%) (Figure 35). After 70 hours, the most noticeable alterations are a decrease in 12:0 (2 to 1%), 18:0 10-methyl (7 to 6%), 18:1 w9c (30 to 22%) and an increase in 14:0 (3 to 4%), 16:0 (13 to 15%), 13:0 *iso* (2 to 4%), 14:0 *iso* (3 to 4%), 15:0 *anteiso* (3 to 4%), 15:0 *iso* (7 to 10%), 16:0 *iso* (3 to 5%), 17:0 *anteiso* (2 to 3%) and 17:0 *iso* (7 to 9%).

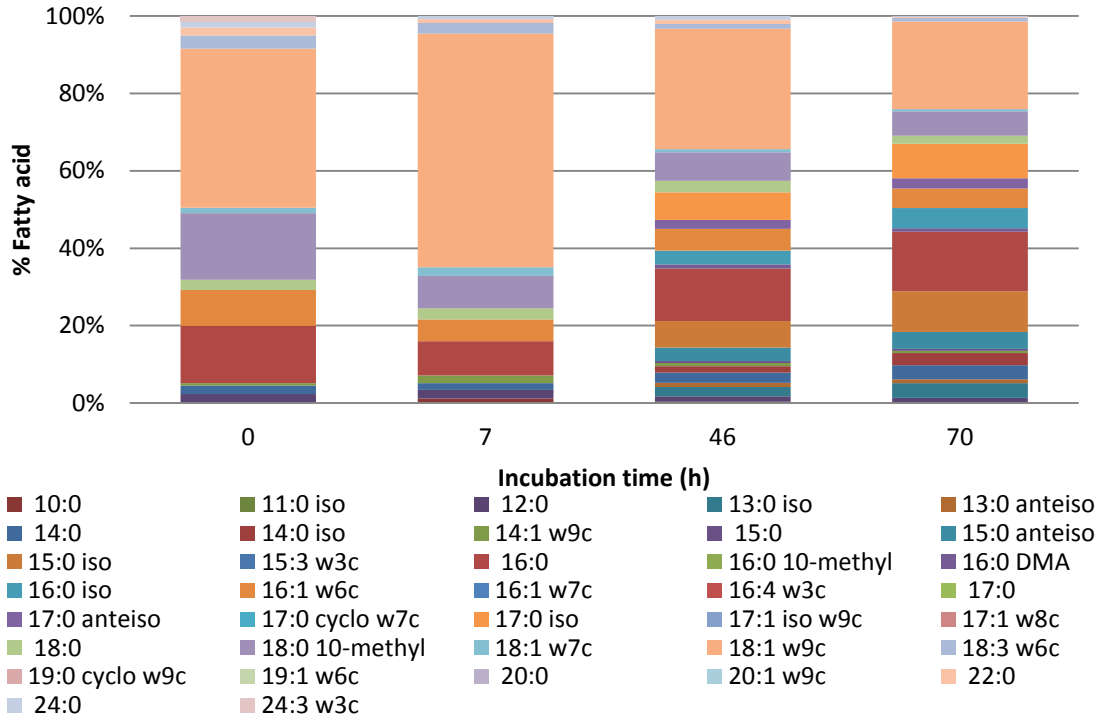


Figure 35 - Percentage of fatty acids present during control conditions, where only MTBE was supplemented to the media.

Under control conditions (Figure 36), MUFA accounted for 52% of the total FA, followed by SSFA (25%), 10-methyl FA (17%) and PUFA (5%). After the addition of 1% MTBE, the saturation degree quickly decreased, and an increase in the content of MUFA (70%) was observed, whilst SSFA (18%), 10-methyl (8%) and PUFA (3%) decreased. After 46 hours, BSFA were produced, and corresponded to 28% of the total FA, together with 7% 10-methyl FA, 23% SSFA, 38% MUFA and 3% PUFA. The amount of BSFA kept increasing until it was 39% after 70 hours. These results are in accordance with the results previously obtained for cells exposed to MTBE (section 3.1), were there was a quick decrease in the saturation degree, followed by a concomitant increase. This increase was also verified during cellular adaptation to MTBE.

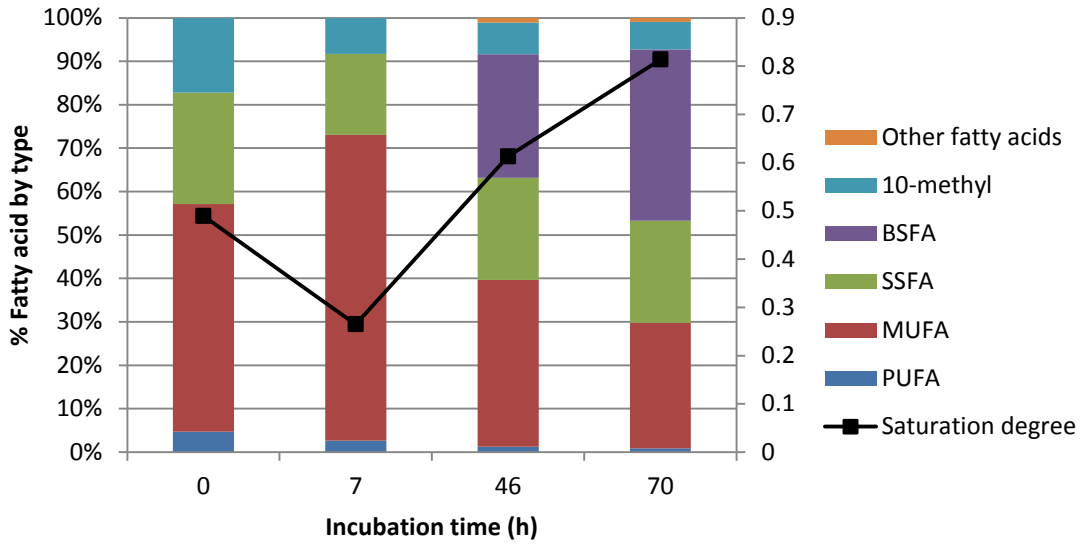


Figure 36 – Percentage of fatty acids by type of cells exposed to 1% MTBE.

When observing the fatty acid profile of cells exposed to MTBE and antibiotics/EPIs, the cellular response is diverse. Nonetheless, the response to either type of compounds results in an immediate decrease in the saturation degree of cells, which tends to increase throughout time.

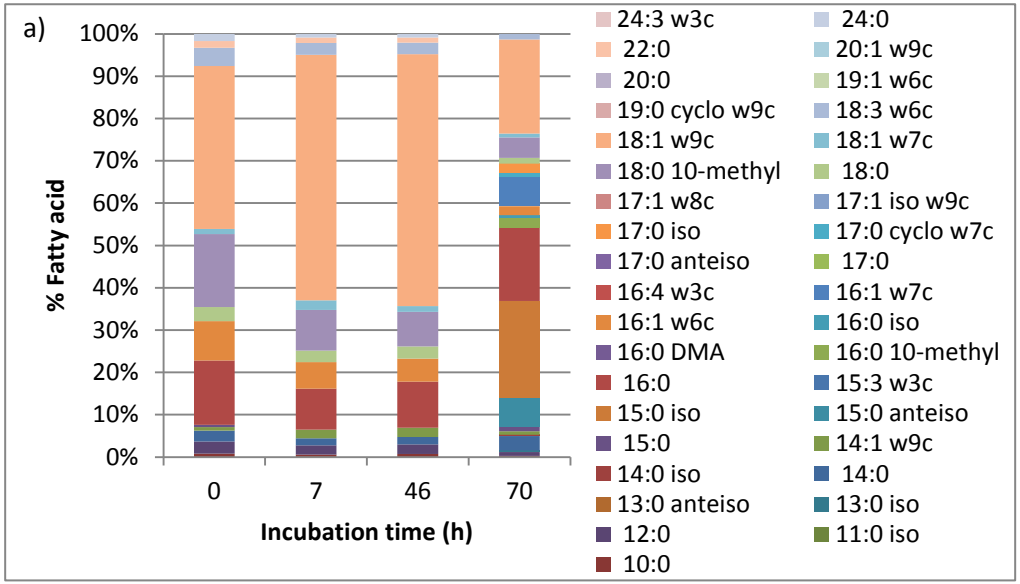
In cells exposed to MTBE and ½ MIC LEVO, there was an increase in the amount of MUFA (48 to 69%) and a decrease in the amount of SSFA (27 to 19%), 10-methyl FA (17 to 10%) and PUFA (4 to 3%) (Figure 37 b)). This increase in the content of MUFA was mainly due to an increase in 18:1 w9c, from 37 to 58%, since other MUFA experienced a small decrease, such as 16:1 w6c (9 to 5%) (Figure 37 a)). The presence of BSFA was essentially due to the production of 15:0 *anteiso* (7%), 15:0 *iso* (22%) and 17:0 *iso* (2%).

In cells exposed to MTBE and ½ MIC TEICO, cells start growing very fast. It was possible to observe that at early hours of exposure there was an increase in MUFA (52 to 71%) and a decrease in SSFA (25 to 17%), 10-methyl FA (16 to 8%) and PUFA (5 to 2% (Figure 38 b)). This increase in MUFA was mainly due to 18:1 w9c that increases from 41 to 61%. There was also a decrease in 16:0 (14 to 8%) and 16:1 w6c (9 to 5%) (Figure 38 a)). After 46 hours, other fatty acids type appeared as 13% of the total FA. The production of 17:0 cyclo w7c accounted for 12% of the other FA produced.

When cells were grown in 1% MTBE and ½ MIC of THIO, the lag phase lasted 54 hours. There was a slight decrease in saturation degree after 7 hours of growth, marked by the increase in the content of MUFA (44 to 56%) and the decrease in SSFA (28 to 25%) and 10-methyl FA (20 to 15%) (Figure 40 b)). After 70 hours of incubation, BSFA were synthesized and represented 24% of the total FA. These were essentially 13:0 *iso* (2%), 15:0 *anteiso* (3%), 15:0 *iso* (8%), 16:0 *iso* (15%), 17:0 *anteiso* (2%) and 17:0 *iso* (4%). SSFA accounted for 23% of the total FA, MUFA 41% and 10-methyl FA represented only 7%.

In cells grown with MTBE and OME, an increase in the content of MUFA (52 to 67%) due to a higher amount of 18:1 w9c and a decrease in SSFA (26 to 20%) mainly associated with 16:0 was perceived

(Figure 41 a)). Between 7 and 70 hours of incubation, there was an increase in the saturation degree of cells due to a decrease in the content of MUFA (67 to 53%) and an increase in SSFA (20 to 27%) and 10-methyl FA (10 to 16%). During this period, there was an increase in 16:0 (10 to 19%), 16:1 w6c (7 to 10%) and a decrease in 18:1 w9c (56 to 40%).



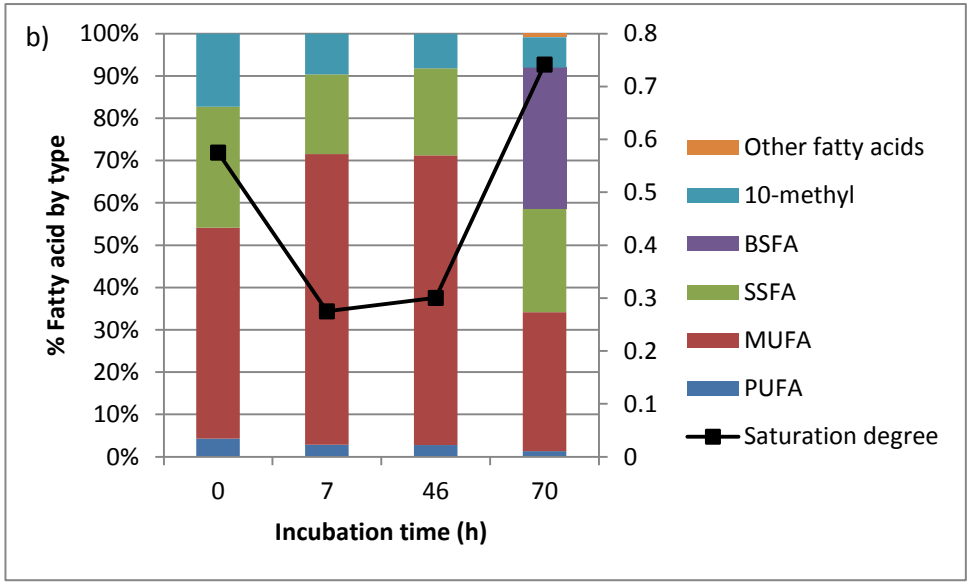
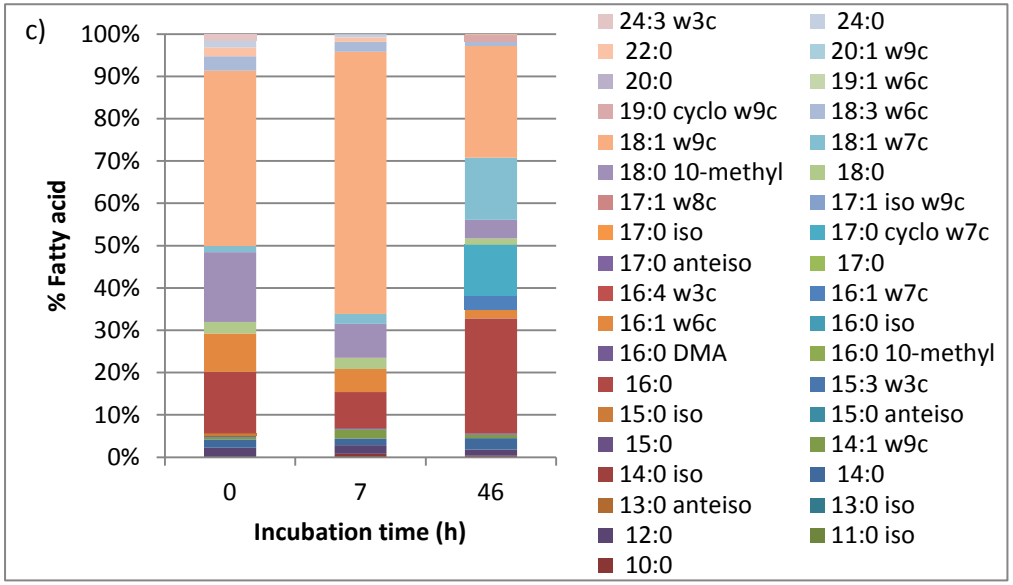


Figure 37 - Fatty acid composition of cells grown in 1%MTBE + 1/2 MIC LEVO, a) by fatty acid and b) by type.



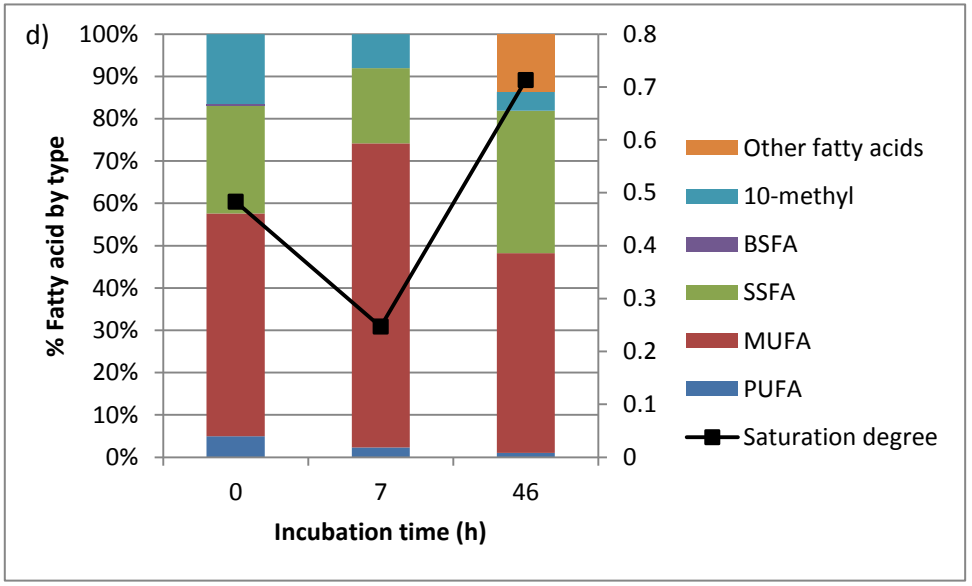
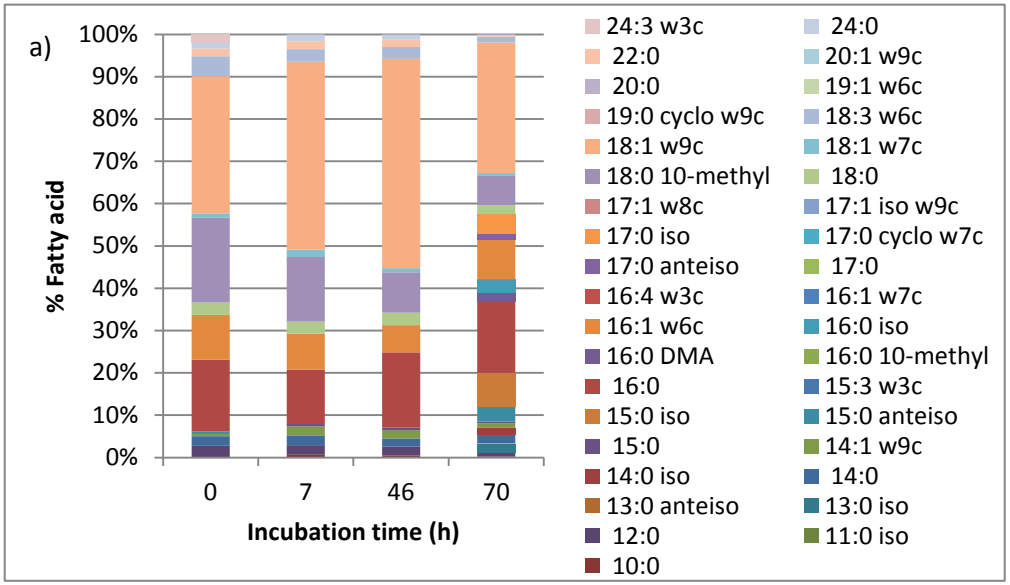


Figure 38 - Fatty acid composition of cells grown in 1%MTBE + 1/2 MIC TEICO, a) by fatty acid and b) by type.



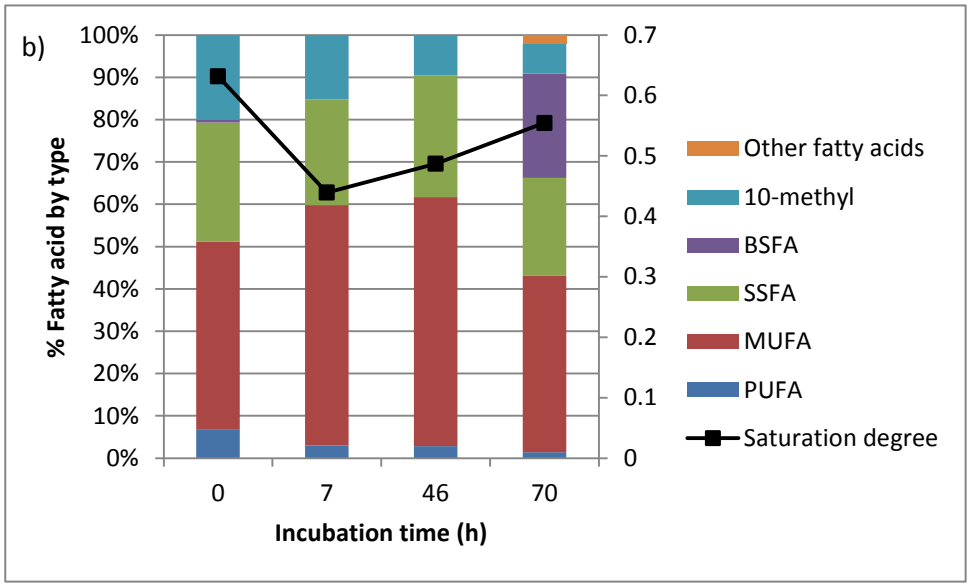
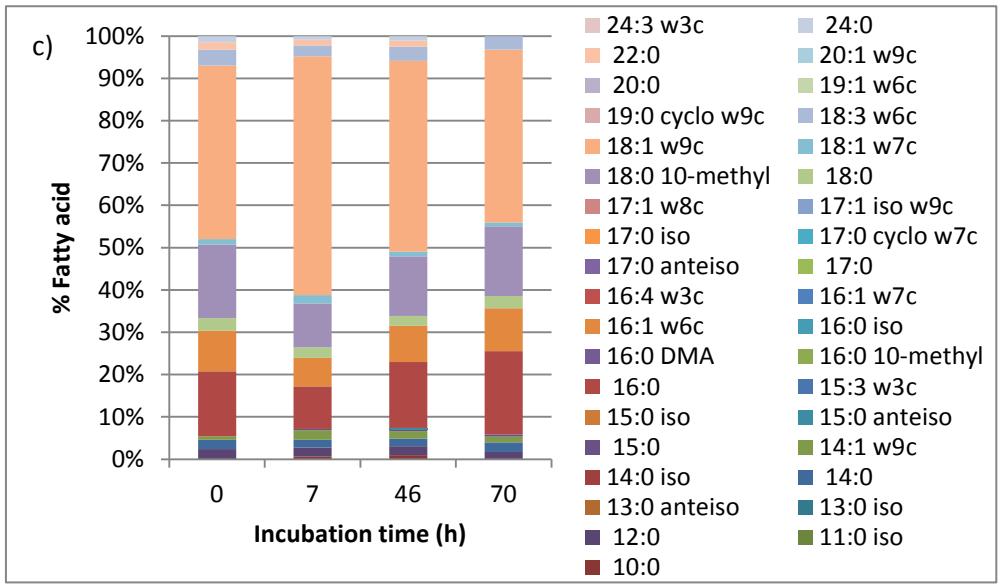


Figure 39 - Fatty acid composition of cells grown in 1%MTBE + 1/2 MIC THIO, a) by fatty acid and b) by type.



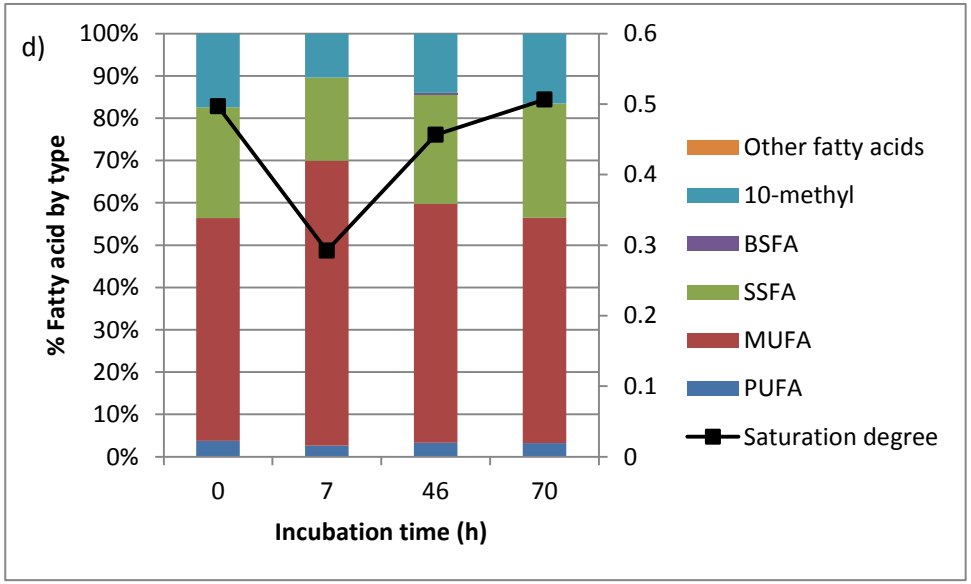


Figure 40 - Fatty acid composition of cells grown in 1%MTBE + 1/2 MIC OME, a) by fatty acid and b) by type.

In non-adapted cells exposed to ethanol and ½ MIC of antibiotics/EPIs the fatty acid composition was also analyzed (Figure 41). After 46 hours, there was an increase in 16:0 (15 to 25%), 16:1 w6c (10 to 11%) and a decrease in 12:0 (3 to 2%), 18:0 10-methyl (19 to 13%), 18:1 w9c (37 to 33%) and 18:3 w6c (4 to 3%). After 70 hours, the amount of 18:1 w9c kept decreasing, until it was 30%, but the amount of 16:1 w6c increased until it was 15%.

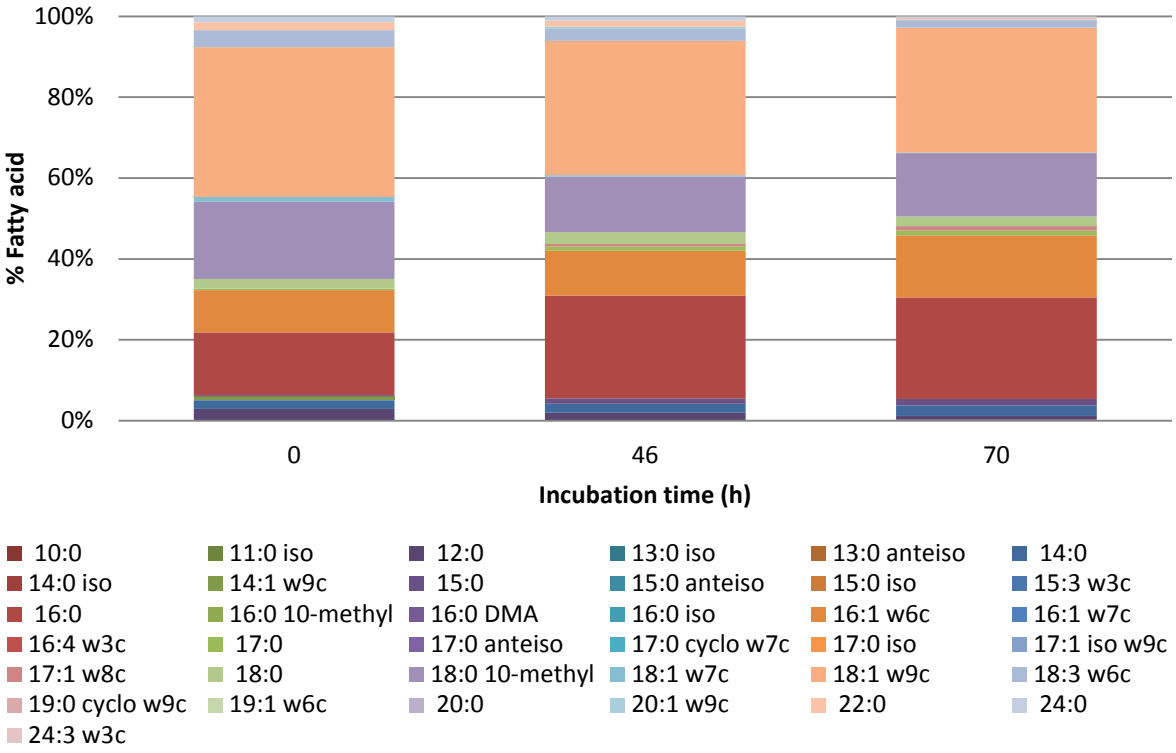


Figure 41 - Percentage of fatty acids present during control conditions, where only ethanol was supplemented to the media.

Under control conditions, where only ethanol was added to the culture, MUFA were the major type of FA present, accounting for 49% of the total FA content, followed by SSFA (27%), 10-methyl FA (19%) and PUFA (4%) (Figure 42). After 46 hours, the saturation degree of cells increased, mainly due to an increase in SSFA (until 37%) and a decrease in MUFA (until 45%) and 10-methyl FA (until 13%). Between 46 and 70 hours, there was a decrease in SSFA (37 to 34%) and an increase in MUFA (45 to 47%).

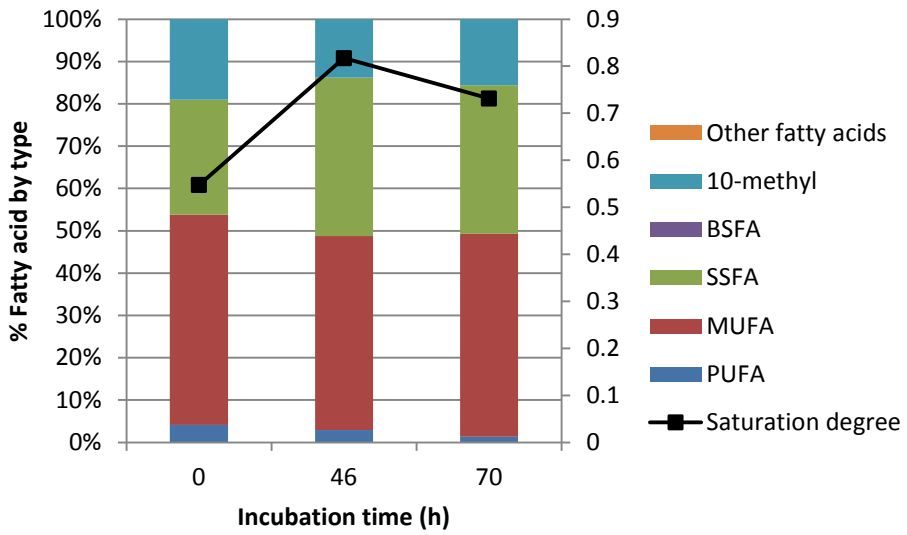


Figure 42 – Percentage of fatty acids by type of cells exposed to 5% ethanol.

When cells were exposed both to ethanol and ½ MIC LEVO, there was a fast decrease in the saturation degree of cells after 7 hours (Figure 43 b)). During this period, there was a decrease in 16:0 (15 to 11%), 16:1 w6c (10 to 7%), and an increase in 18:1 w9c (38 to 40%) (Figure 43 a)). After 70 hours, BSFA were produced, in a total amount of 11%, being essentially composed by 15:0 *anteiso* (9%) and 15:0 *iso* (2%), and 20:1 w9c was synthesized, accounting for 14% of the total FA.

In cells grown in the presence of both ethanol and TEICO, there was an increase in MUFA (48 to 63%), and a decrease in SSFA (29 to 21%) and 10-methyl FA (18 to 12%). Similar to the previous cases, this was due to an increase in 18:1 w9c and a decrease in 16:0 and 16:1 w6c (Figure 44 a)). Other FA were produced after 46 hours, where they were 9% of the total FA content, increasing until a final amount of 23% after 70 hours. These were mainly 17:0 cyclo w7c (21%) and 19:0 cyclo w9c (2%).

In cells exposed both to ethanol and THIO, there was a decrease in the saturation degree of cells (Figure 45 b)). This was due to a continuous increase in MUFA (43 to 69%) and a decrease in SSFA (30 to 17%), 10-methyl FA (21 to 10%) and PUFA (5 to 2%), throughout the incubation time considered. In this case, there was a decrease in 16:0 (19 to 9%), 16:1 w6c (11 to 7%), 18:0 (3 to 2%), 18:0 10-methyl (21 to 10%), 18:1 w7c (2 to 1%), 18:3 w6c (5 to 3%) and an increase in 14:1 w9c (1 to 8%) and 18:1 w9c (29 to 54%) (Figure 45 a)).

In the presence of ethanol and ½ MIC OME, the amount of MUFA increased (50 to 60%), and SSFA decreased (30 to 22%), essentially due to a decrease in 16:0 (14 to 12%) and an increase in 18:1 w9c (39 to 48%) (Figure 46 a)). After 46 hours, this trend was inverted, and the amount of MUFA decreased until it is 48% of the total FA, whilst SSFA increased until it is 33%. This led to an increase in the saturation degree of cells.

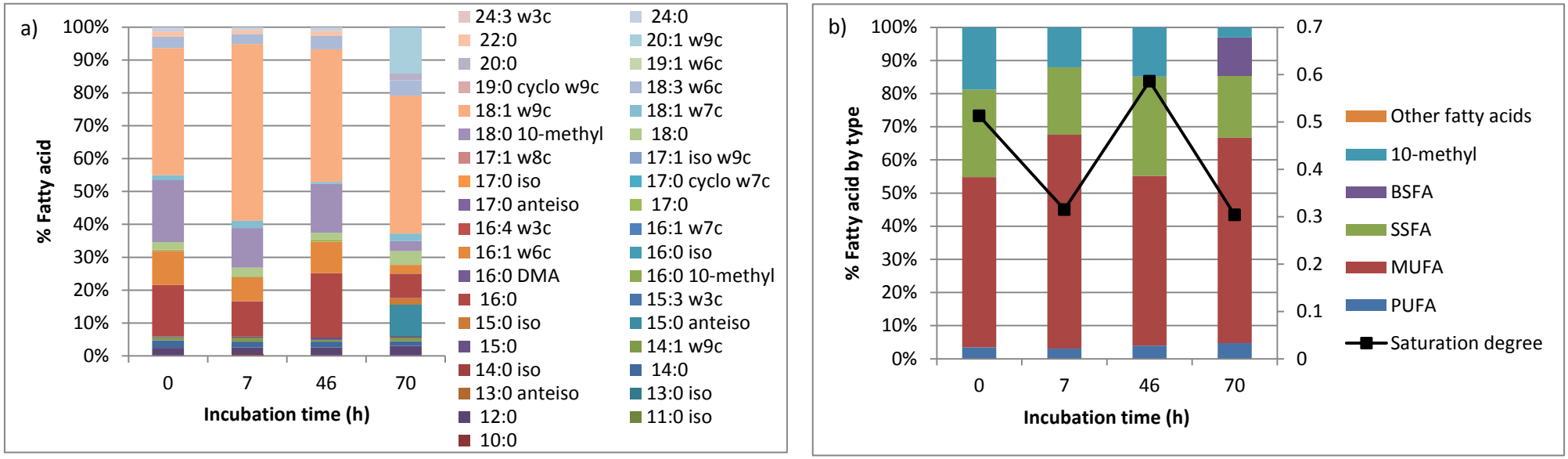


Figure 43 - Fatty acid composition of cells grown in 5% ethanol + 1/2 MIC LEVO, a) by fatty acid and b) by type.

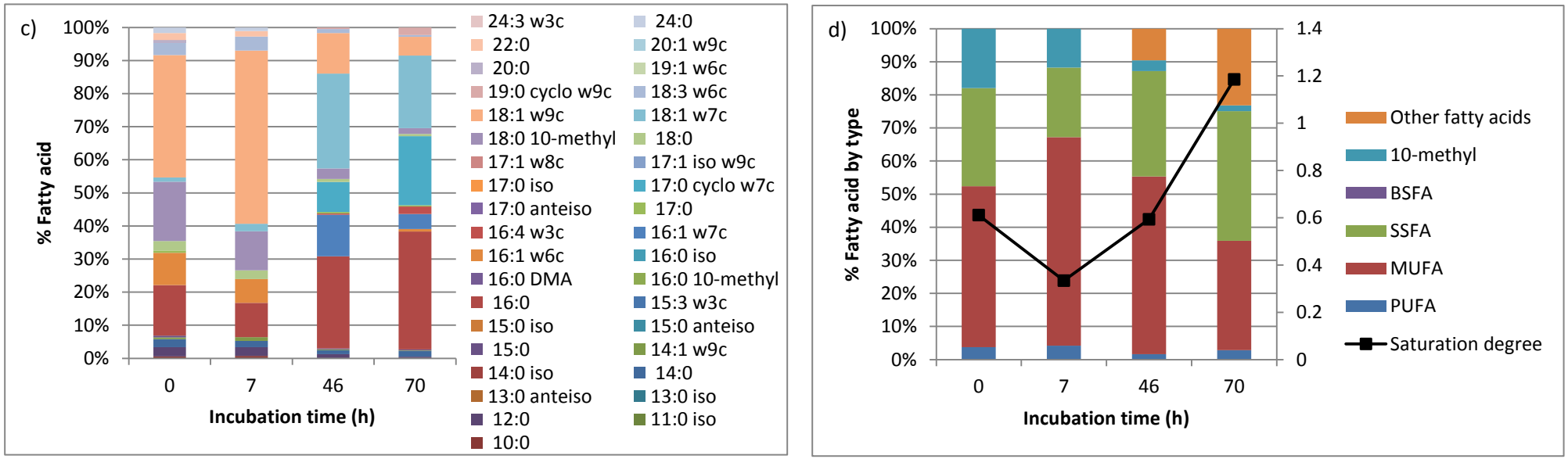


Figure 44 - Fatty acid composition of cells grown in 5% ethanol + 1/2 MIC TEICO, a) by fatty acid and b) by type.

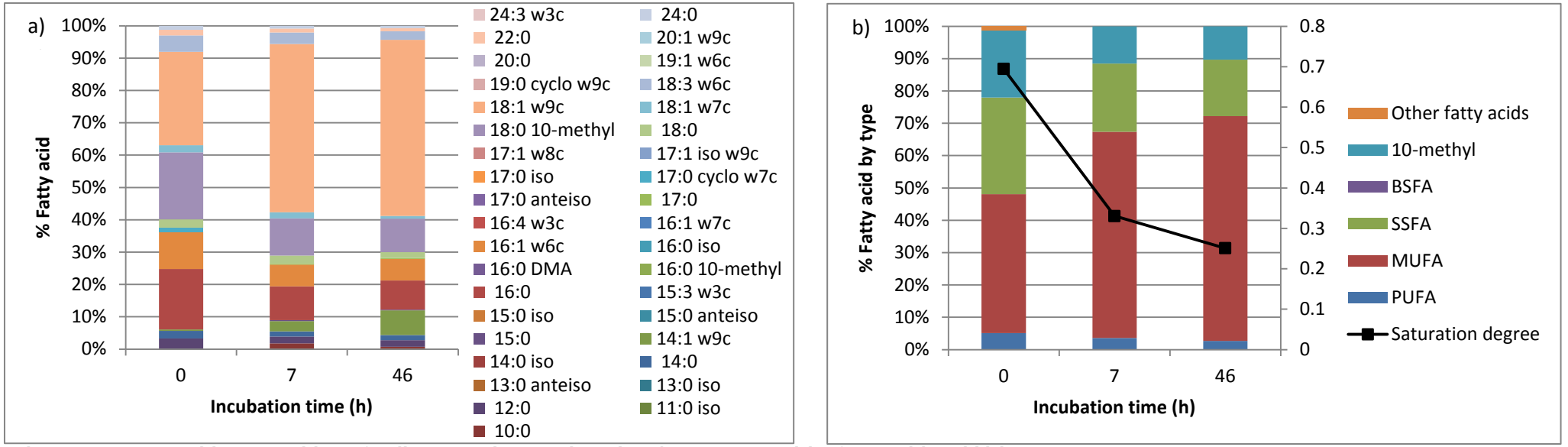


Figure 45 - Fatty acid composition of cells grown in 5% ethanol + 1/2 MIC THIO, a) by fatty acid and b) by type.

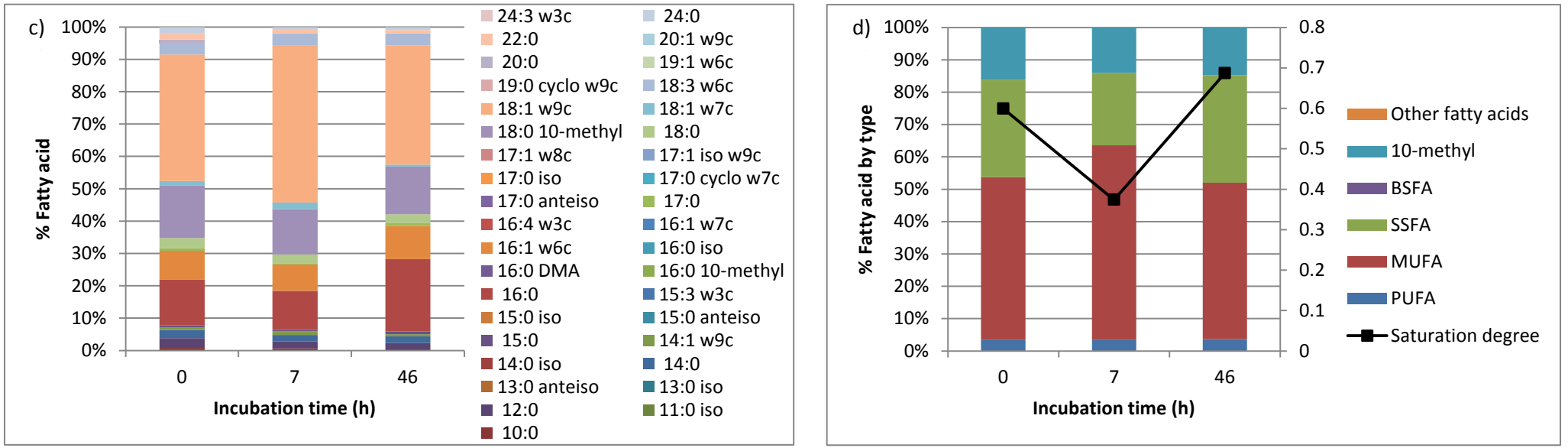


Figure 46 - Fatty acid composition of cells grown in 5% ethanol + 1/2 MIC OME, a) by fatty acid and b) by type.

3.4.2 Solvent-adapted cells grown in ½ MIC of antibiotics and EPIS

A cellular suspension of MTBE-adapted cells (OD = 1.19) was used to inoculate MH media with 1% MTBE and ½ MIC of antibiotics and EPIs. OD values of 11.1 and 9.3 were obtained in 1% MTBE and 1% MTBE + ½ MIC OME, respectively, after 26 hours of growth (Figure 47). Cells in the presence of MTBE +1/2 MIC LEVO had a small lag phase until 20 hours of growth, achieving a maximum OD of 5.1. In 1% MTBE + ½ MIC TEICO and THIO, lag phase lasted until approximately 43 hours, and ODs of 5.83 and 3.27 were achieved.

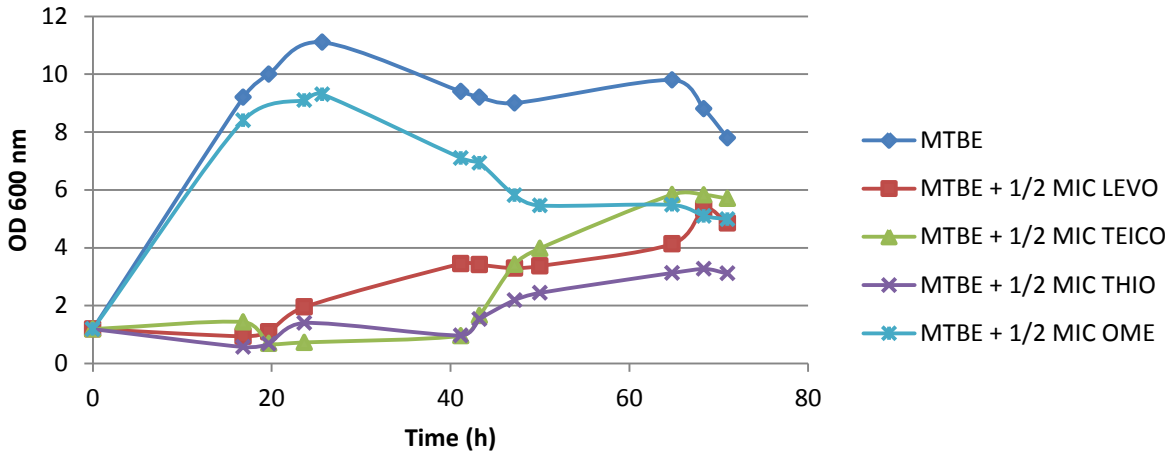


Figure 47 - MTBE adapted cells re-grown in the presence of 1% MTBE and 1/2 MIC of antibiotics and EPIs.

Ethanol-adapted cells (OD = 0.53) were grown in the presence of 5% ethanol and ½ MIC of antibiotics and EPIs (Figure 48). Both in control conditions and in 5% ethanol + ½ MIC LEVO, ODs of 2.5 were obtained. In the other cases almost no growth occurred, and in both cases where TEICO and THIO were present maximum growth was achieved after 26 hours (OD= 0.85 and 0.95, respectively). In cells grown with ethanol and OME, maximum growth was achieved after 29 hours, and a maximum OD of 0.88 was measured.

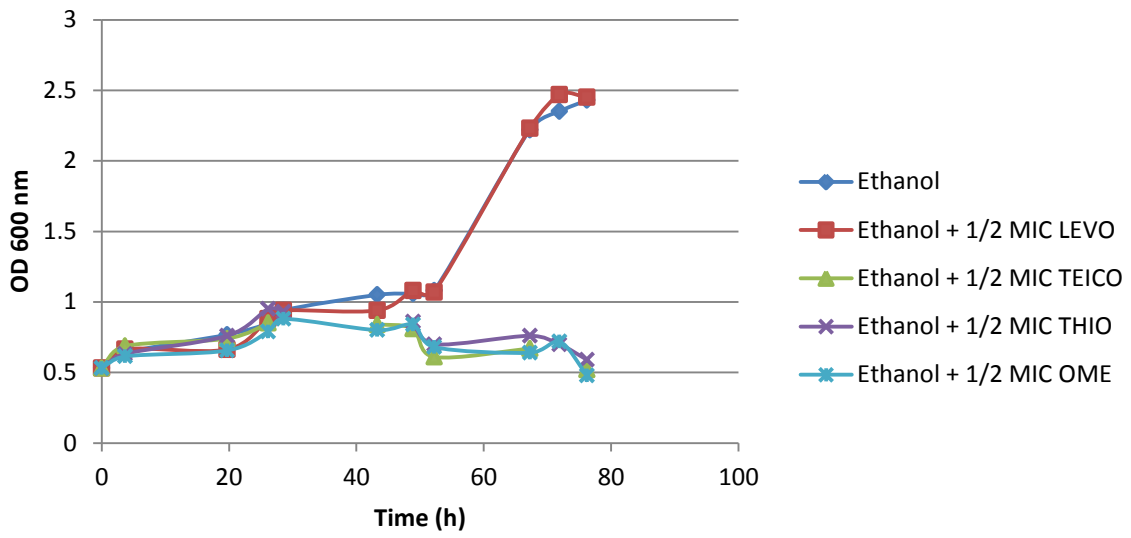


Figure 48 - Ethanol adapted cells re-grown in the presence of 5% Ethanol and 1/2 MIC of antibiotics and EPIs.

MTBE-adapted cells were observed using fluorescence microscopy, after 45 hours of incubation. In the presence of 1% MTBE, dark vesicles inside the cells were enhanced by the fluorophore (Figure 49 a)). Also, the presence of a blur surrounding the cells pointed to the possible production of EPS by *M. vaccae* in the presence of MTBE, being particularly visible in MTBE+OME (Figure 49 c and d)). In order to understand if EPS was being produced, Nile red staining was used to evidence a matrix of possible lipidic nature surrounding the cells. The mycolates group, where *Mycobacterium* is inserted, are known to produce several types of trehalose containing glycolipids (Franzetti, Gandolfi, Bestetti, Smyth, & Banat, 2010).

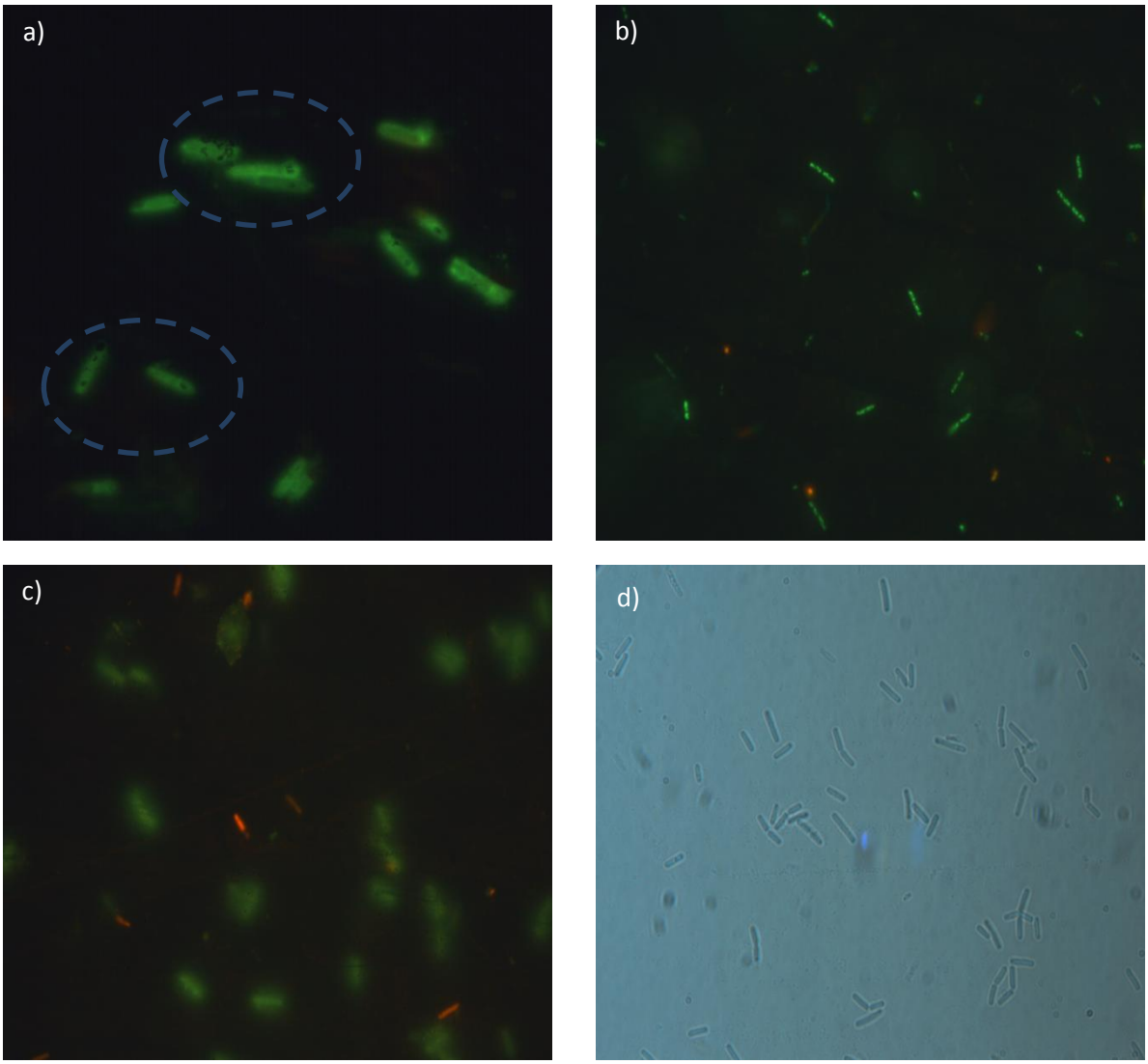


Figure 49 - MTBE-adapted cells in the presence of MTBE (a), MTBE+TEICO (b) and MTBE+OME (c and d).

Surprisingly, the Nile red did not stain the EPS, but disclosed the presence of lipid bodies inside the cells (Figure 50). Storage lipid compounds are a class of vital fatty acid derived compounds and can thus be used as an endogenous source of carbon and energy, or as a sink for reducing equivalents (Alvarez & Steinbüchel, 2003; Wältermann & Steinbüchel, 2005). The accumulation of these droplets may be a result of homeoviscous adaptation to environmentally stressful conditions, such as solvent exposure. Accumulation of triacylglycerols as storage compounds during growth on hydrocarbons was already observed for members of the *Mycobacterium* genus (M. Alvarez, 2003).

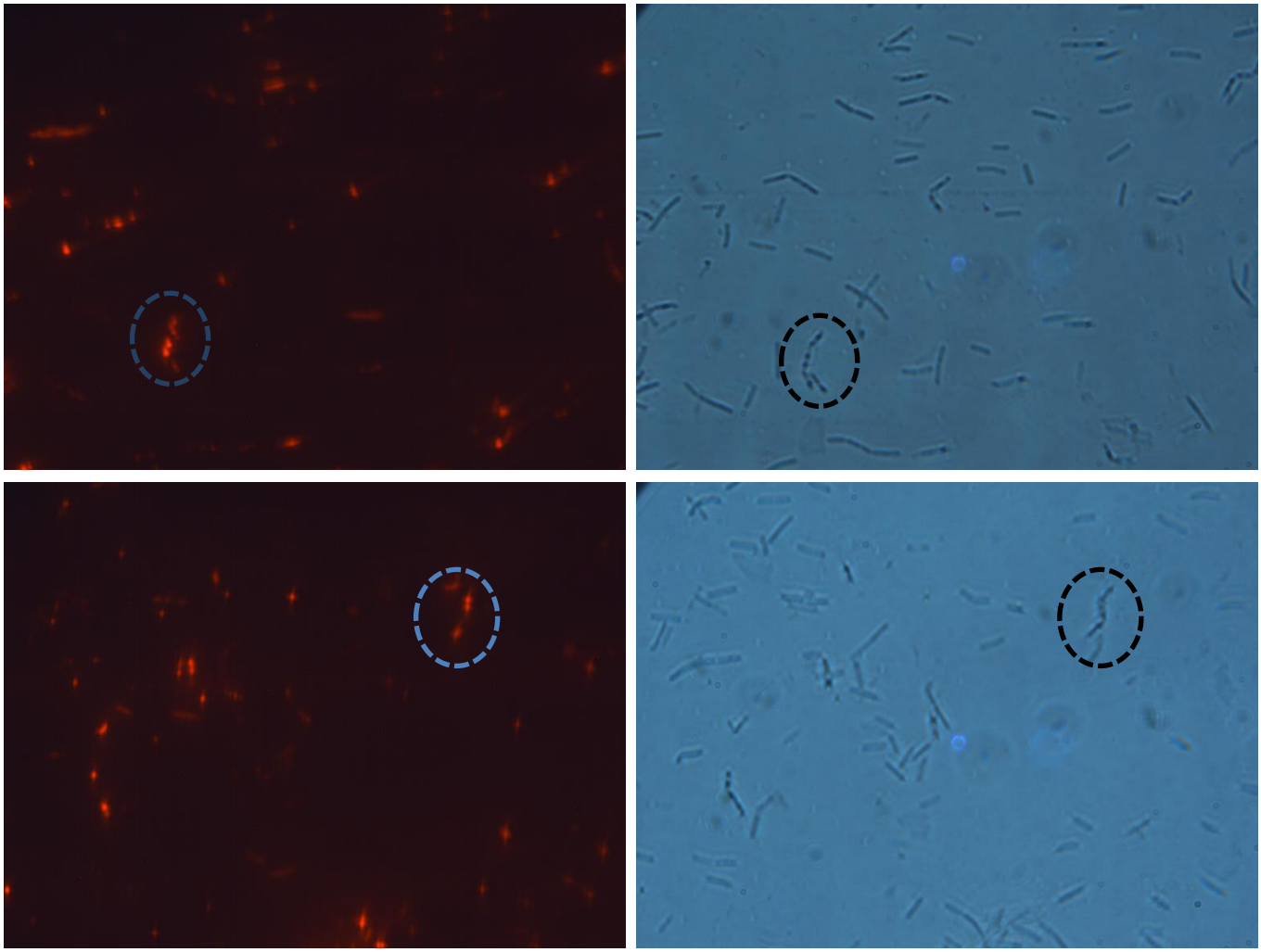


Figure 50 - Nile red staining of MTBE-adapted cells in the presence of 1% MTBE, evidencing lipid vesicles inside the cells.

In order to test if the EPS observed was composed by extracellular DNA, the supernatant was collected and $OD_{260-280}$ was measured. When plotting the results against an aliquot of MH media used as blank, there was an overlap between the plots, possibly due to the contribution of the media components at the same wavelengths as the DNA, hampering the confirmation of the nature of the EPS produced by the cells (data not shown).

In MTBE-adapted cells re-grown in the presence of fresh media supplemented with 1% MTBE, the most common FA were 13:0 *iso* (2%), 14:0 (2%), 14:0 *iso* (2%)15:0 *anteiso* (2%), 15:0 *iso* (12%), 16:0 (7%), 17:0 *iso* (7%), 18:0 (2%), 18:1 w7c (3%) and 18:1 w9c (51%) (Figure 51). Throughout time, there was a particular increase in the amount of 17:0 *iso* (7 to 9%), 15:0 iso (12 to 16%)) and a decrease in 18:1 w9c (51 to 42%).

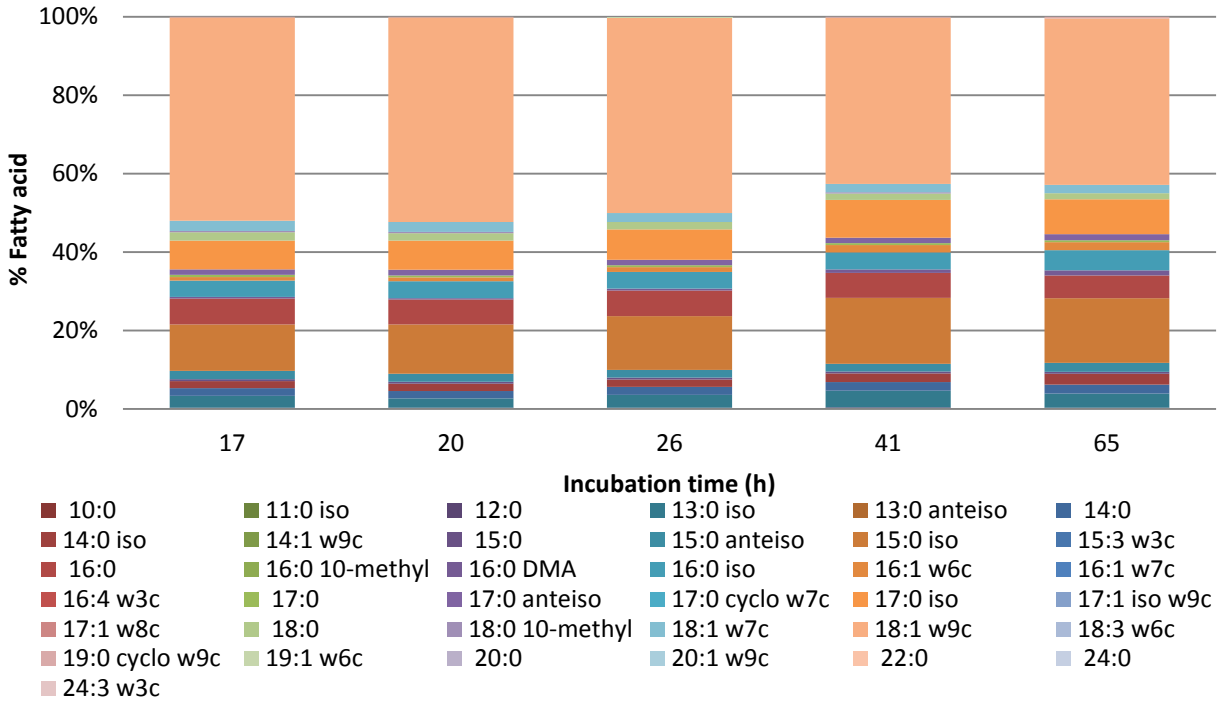


Figure 51 – Percentage of fatty acids in MTBE-adapted cells exposed to 1% MTBE.

The major types of FA present were MUFA (55%), BSFA (31%) and SSFA (12%) (Figure 52). Throughout time, there was a continuous increase in BSFA (from 31 to 40%) and a decrease in MUFA (55 to 46%). After this period, the saturation degree decreased between 41 and 63 hours, due to a slight decrease in the amount of SSFA (12 to 11%).

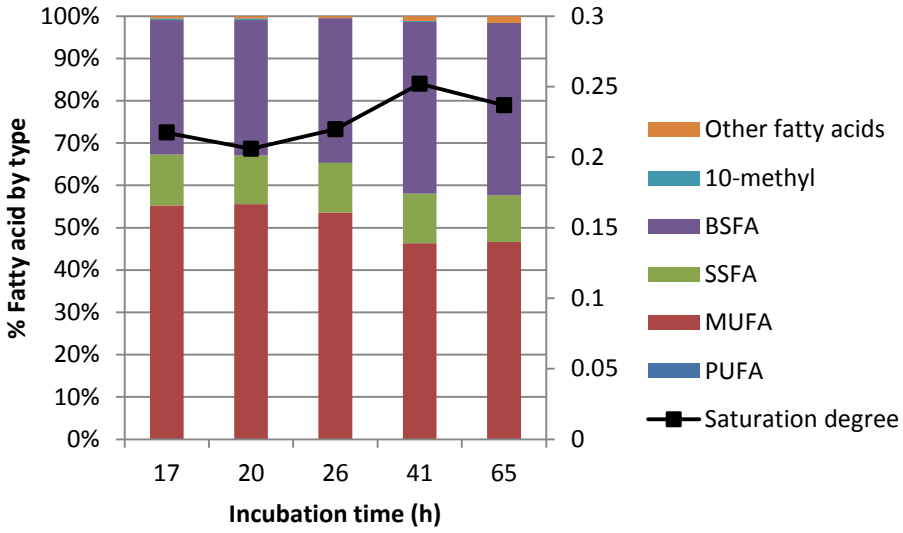


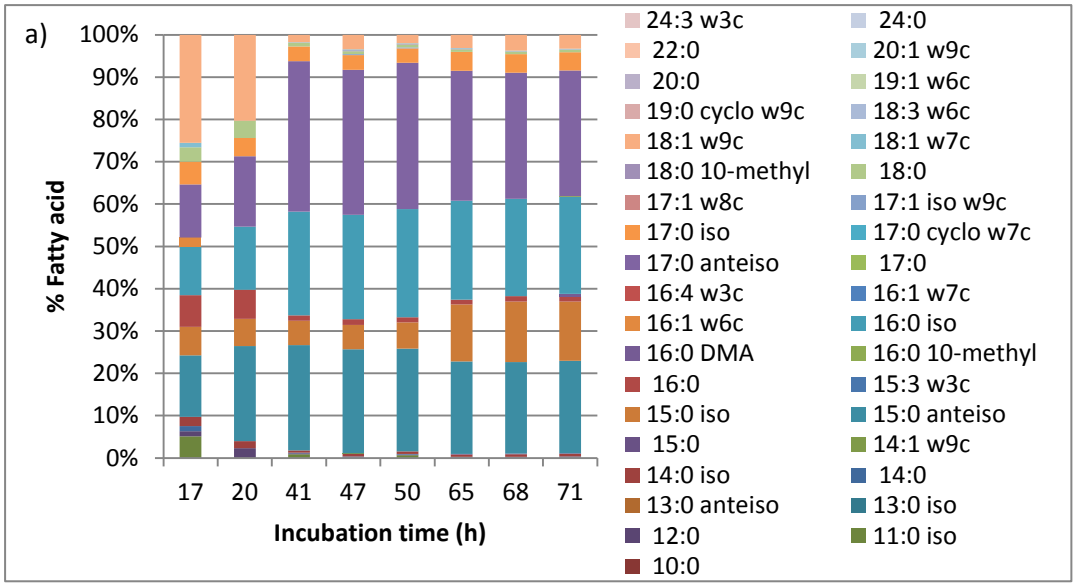
Figure 52 – Percentage of fatty acids by type in MTBE-adapted cells exposed to 1% MTBE.

When MTBE-adapted cells were grown in 1% MTBE and ½ MIC LEVO, after 17 hours, BSFA represented 58% of the total FA content, reaching values always higher than 90% in the following hours of incubation (Figure 54 b)). This was due to an increase in the amount of 15:0 *anteiso* (15 to 25%), 15:0 *iso* (6 to 14%), 16:0 *iso* (11 to 23%), 17:0 *anteiso* (13 to 29%), and a decrease in 17:0 *iso* (5 to 4%) and 11:0 *iso* (5 to 1%) (Figure 54 a)).

In MTBE-adapted cells exposed to TEICO and 1% MTBE, there was a decrease in the saturation degree until 20 hours of exposure, but this value kept increasing throughout time (Figure 53 b)). After 17 hours the most common FA were 11:0 *iso* (7%), 12:0 (2%), 14:0 (2%), 14:0 *iso* (4%), 15:0 *anteiso* (2%), 15:0 *iso* (12%), 16:0 (12%), 16:0 *iso* (7%), 16:1 w6c (3%), 17:0 *anteiso* (2%), 17:0 *iso* (11%), 18:0 (5%) and 18:1 w9c (30%) (Figure 53 a)). After 41 hours, until the rest of the incubation period, the amount of BSFA kept increasing (34 to 42%), same as SSFA (27 to 29%), and MUFA decreased (39 to 25%). There was a huge decrease in 18:1 w9c FA, from 33 to 9%.

In cells exposed to 1% MTBE and THIO, there was a decrease in the content of SSFA (20 to 15%) and BSFA (32 to 18%) and an increase in MUFA (48 to 66%), in the first 41 hours of exposure (Figure 55 b)). During this period, there was a decrease in 14:0 *iso* (3 to 1%), 16:0 *iso* (5 to 2%), 17:0 *iso* (8 to 3%), and an increase in 18:1 w9c (42 to 59%) (Figure 55 a)). In the end, BSFA were the major FA present (45%), followed by SSFA (25%). The amount of 15:0 *anteiso* (2 to 6%), 15:0 *iso* (9 to 32%), 16:1 w7c (3 to 9%) kept increasing, while 18:0 (3 to 1%) and 18:1 w9c (59 to 10%) kept decreasing.

In the presence of both 1% MTBE and OME, cells decreased the amount of MUFA (50 to 41%) in the first 41 hours and increased SSFA (15 to 17%) (Figure 56 b)). During this period, other fatty acids represented 5% of the total FA content, being essentially composed by 19:0 *cyclo* w9c (4%). After 65 hours, there was an increase in 15:0 *iso* (12 to 14%), 17:0 *iso* (6 to 7%) and 19:1 w6c appeared, accounting for 5% of the total FA composition of the cells.



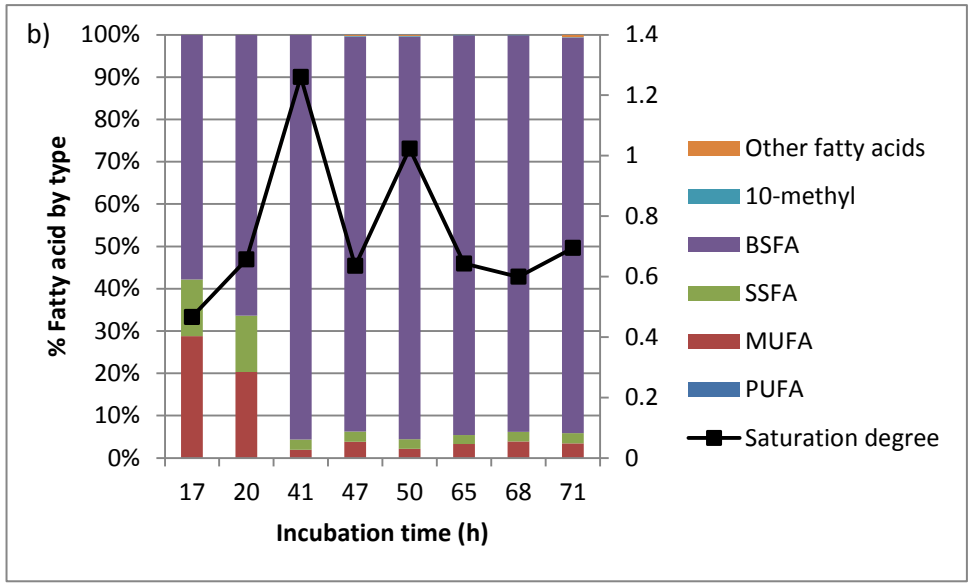
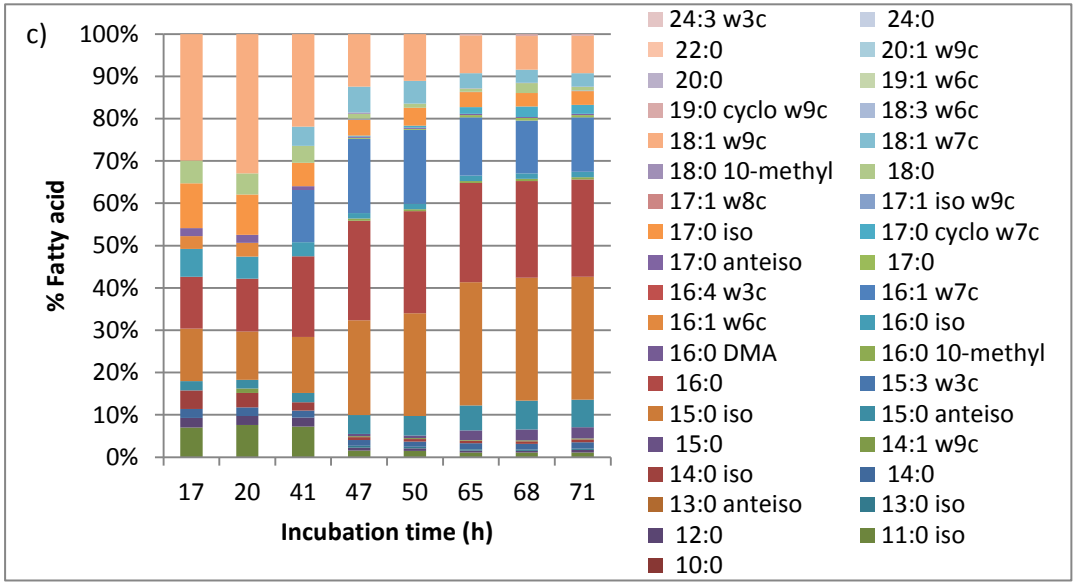


Figure 54 - Fatty acid composition of MTBE-adapted cells grown in 1% MTBE + 1/2 MIC LEVO, a) by fatty acid and b) by type.



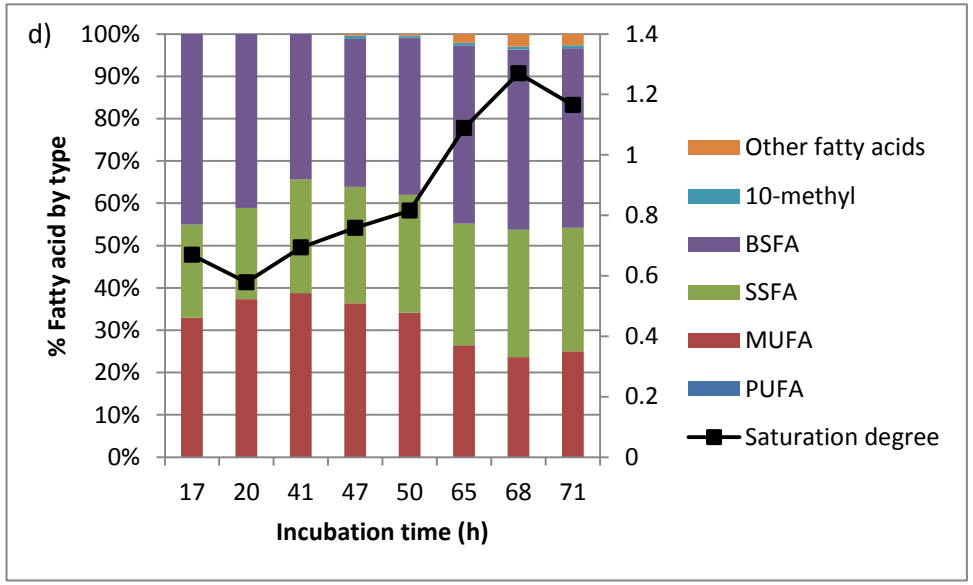


Figure 53 - Fatty acid composition of MTBE-adapted cells grown in 1% MTBE + 1/2 MIC TEICO, a) by fatty acid and b) by type.

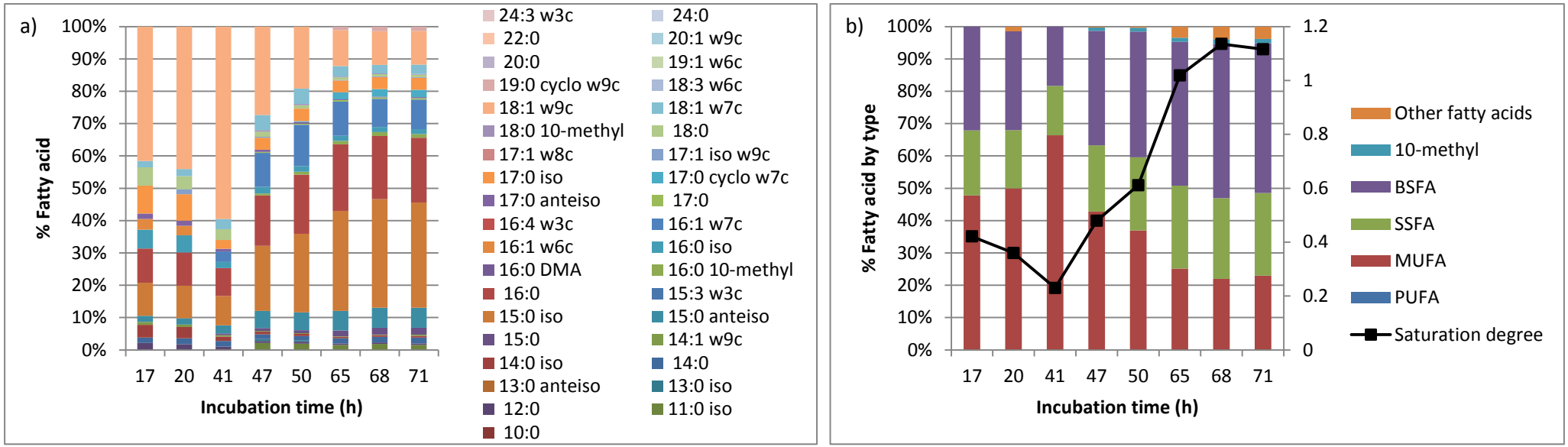


Figure 55 - Fatty acid composition of MTBE-adapted cells grown in 1% MTBE + 1/2 MIC THIO, a) by fatty acid and b) by type.

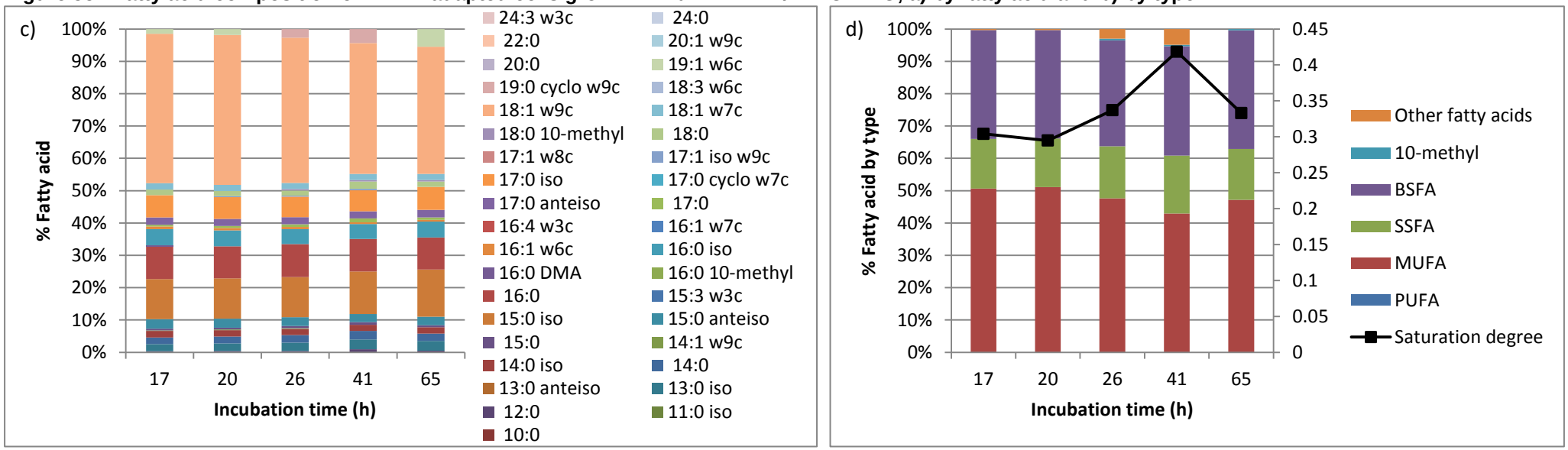


Figure 56 - Fatty acid composition of MTBE-adapted cells grown in 1% MTBE + 1/2 MIC OME, a) by fatty acid and b) by type.

In ethanol-adapted cells, only cells exposed to 5% ethanol and 5% ethanol + LEVO seemed to grow. At time 0, the most common FA were 16:0 (13%), 12:0 (4%), 16:1 w6c (6%), 18:0 (4%), 18:0 10-methyl (6%), 18:1 w9c (50%), and 18:3 w6c (6%) (Figure 57). After 67 hours, there was an increase in 15:0 *anteiso* (1 to 18%) and 20:1 w9c was produced (21%). Also, 18:1 w9c decreased (43 to 32%), together with 16:0 (13 to 5%).

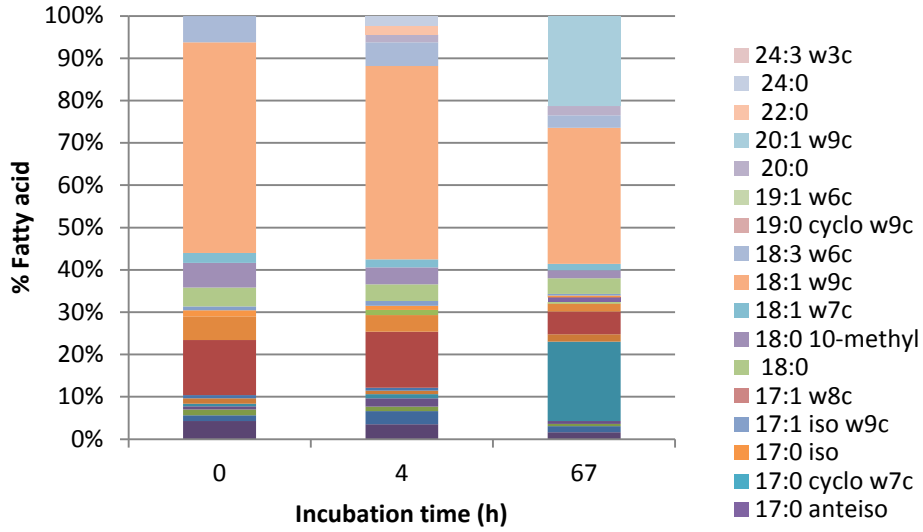


Figure 57 – Percentage of fatty acids present in ethanol-adapted cells grown in 5% ethanol.

When ethanol-adapted cells were re-grown in fresh media with 5% ethanol, the cells were composed by 59% MUFA, 24% SSFA, 7% PUFA, 6% 10-methyl FA and 3% BSFA (Figure 58). After 4 hours of growth, the saturation degree of cells increased, together with the amount of SSFA that represented 31% of the total FA. Throughout time, there was a decrease in the amount of SSFA, to 15%, and BSFA increased up to a content of 22%. There was also a decrease in the saturation degree of cells.

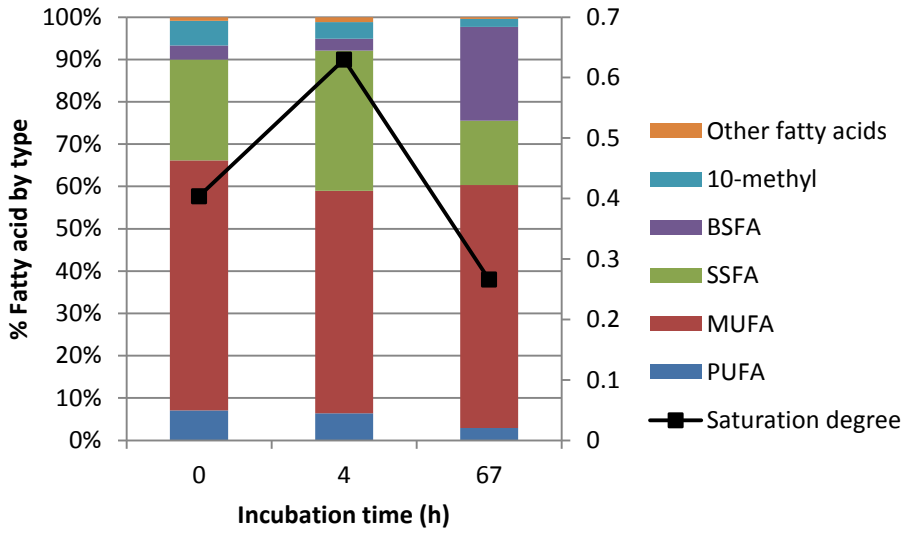


Figure 58 – Percentage of fatty acids by type of ethanol-adapted cells grown in 5% ethanol.

When ethanol-adapted cells were grown in the presence of 5% ethanol + ½ MIC LEVO, the saturation degree decreased throughout time (Figure 59 b)). There was a decrease in 12:0 (4 to 2%), 16:0 (13 to 6%), 16:1 w6c (6 to 2%), 18:0 10-methyl (4 to 2%), 18:1 w9c (52 to 32%) and an increase in 15:0 *anteiso* (1 to 15%), 15:0 *iso* (1 to 2%), and 20:1 w9c FA was produced (20%) (Figure 59 a)).

In the other cases, since no growth occurred, only two time points were analyzed in order to detect differences in a fast response of cells. In the case of cells grown in the presence of TEICO and OME, the saturation degree decreased, whilst in the presence of THIO increased. When grown in ethanol and TEICO, MUFA increased from 61 to 65%, BSFA decreased from 4 to 3% and SSFA decreased from 22 to 21%. The major difference was the increase in 18:1 w9c, from 52 to 57% (Figure 60 a, b)).

In the presence of ethanol and THIO, MUFA increased from 59 to 62%, BSFA disappeared, SSFA increased from 22 to 24% and 10-methyl FA and PUFA remained at the same amount (Figure 61 b)). 17:0 *iso*, 15:0 *iso* and 15:0 *anteiso* (1% each) disappeared after 4 hours of growth, and 18:1 w9c increased from 50 to 53% (Figure 61 a)).

When grown in ethanol and OME, there was an increase in MUFA (56 to 69%), a decrease in BSFA (9 to 3%) and PUFA (8 to 7%) and SSFA remained the same (Figure 62 b)). The increase in MUFA was particularly due to 18:1 w9c, which increased from 48 to 60%. 11:0 *iso* was present at 0 hours, as 5% of the total FA content, and disappeared, together with 18:0 10-methyl (Figure 62 a)).

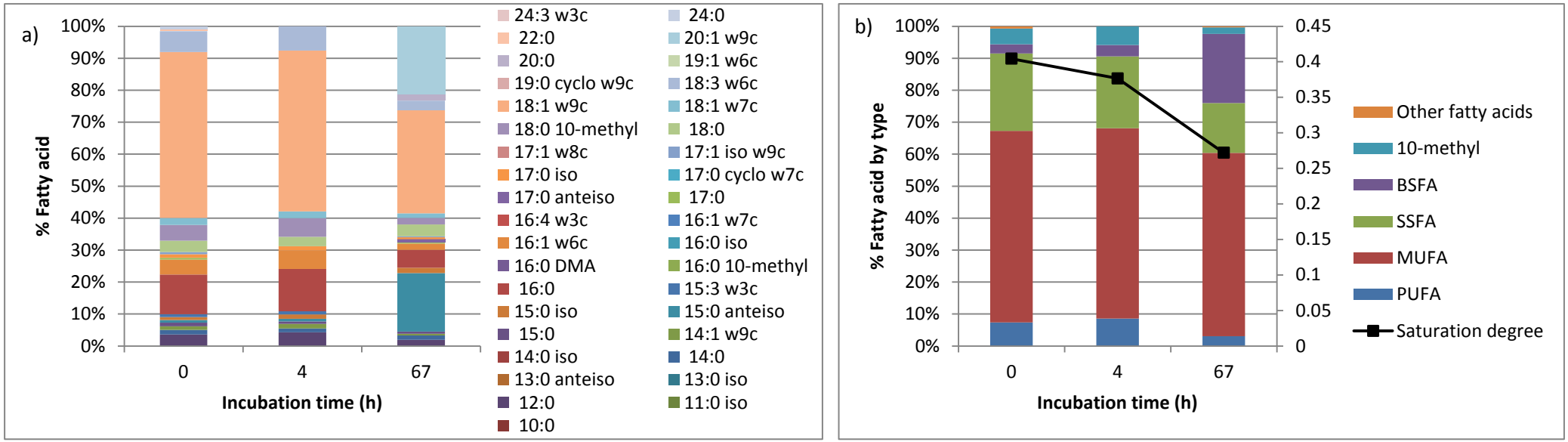


Figure 59 - Fatty acid composition of ethanol-adapted cells grown in 5% ethanol + 1/2 MIC LEVO, a) by fatty acid and b) by type.

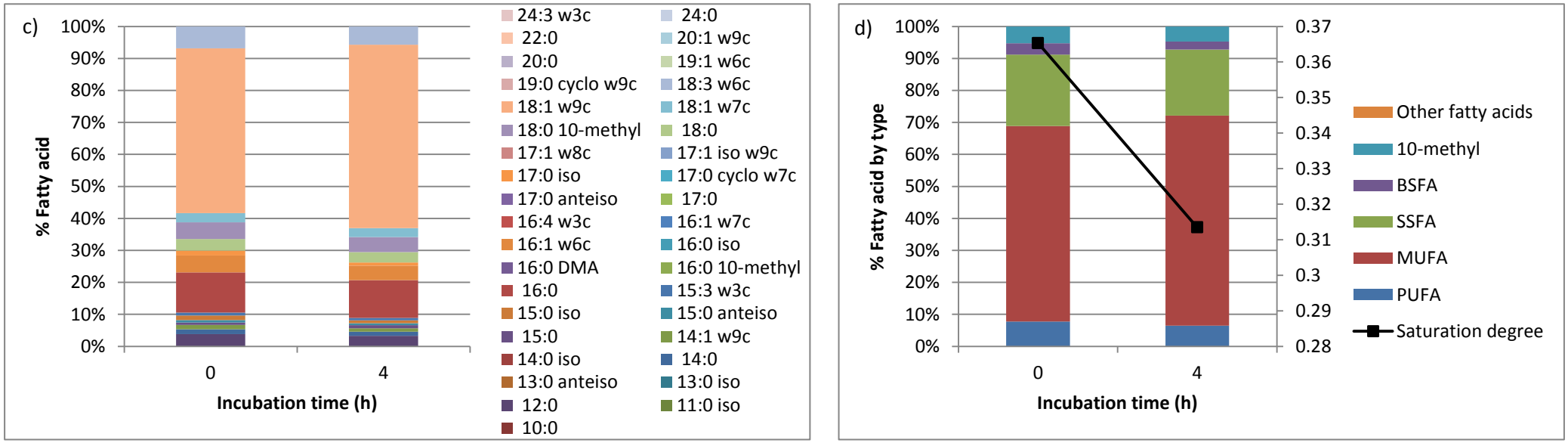
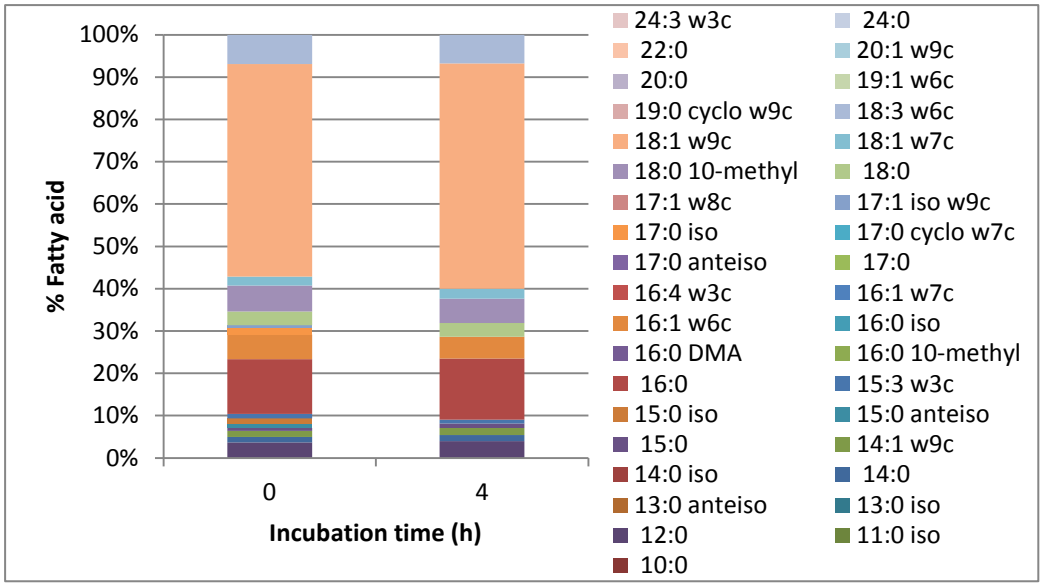


Figure 60 - Fatty acid composition of ethanol-adapted cells grown in 5% ethanol + 1/2 MIC TEICO, a) by fatty acid and b) by type.



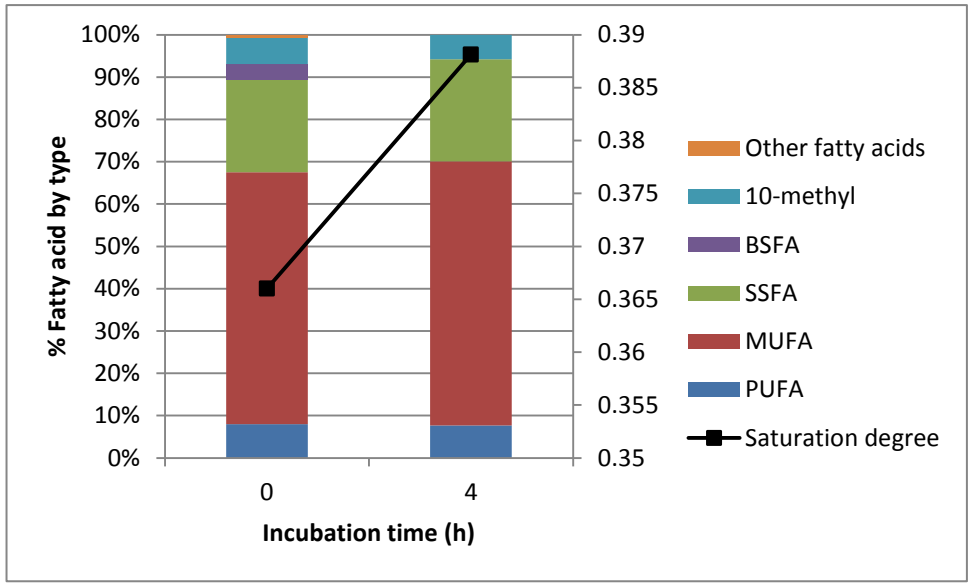
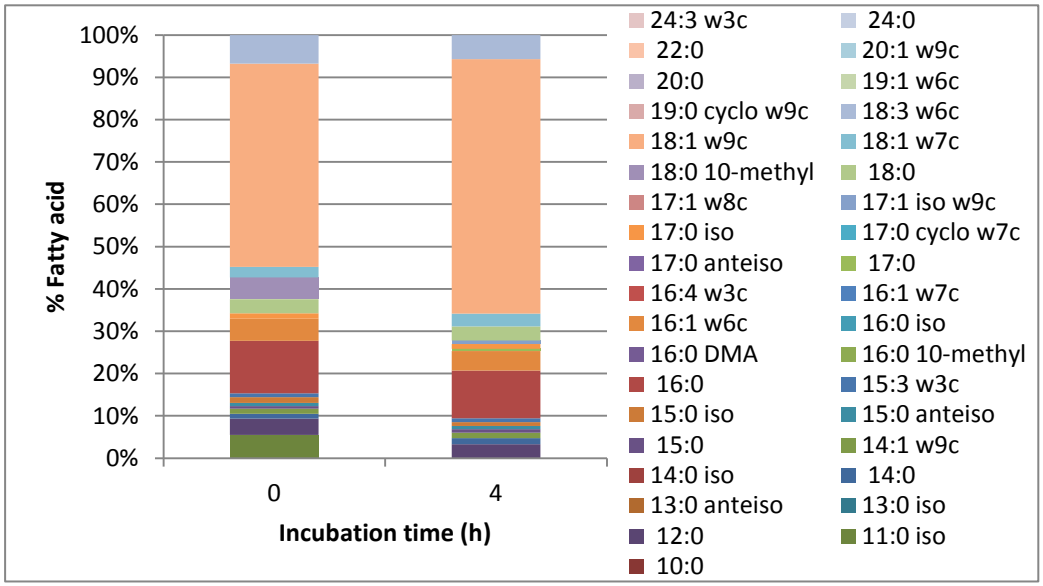


Figure 61 - Fatty acid composition of ethanol-adapted cells grown in 5% ethanol + 1/2 MIC THIO, a) by fatty acid and b) by type.



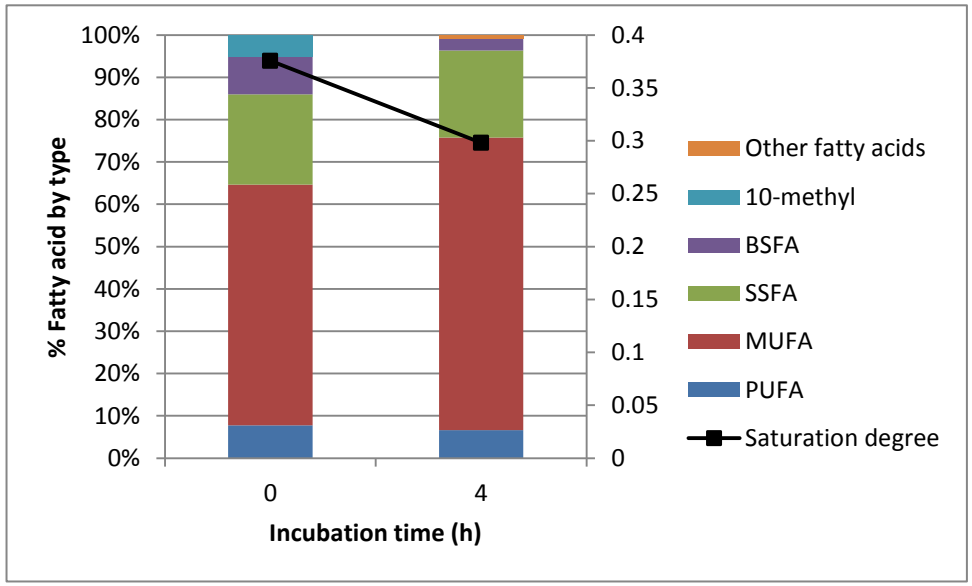


Figure 62 - Fatty acid composition of ethanol-adapted cells grown in 5% ethanol + 1/2 MIC OME, a) by fatty acid and b) by type.

When analyzing the results, many differences were observed between the behavior of solvent-adapted and non-adapted cells, and variability between the cells adapted to different solvents was also detected, essentially due to the additive effect of the organic solvents and the antibiotics/EPIs (Table 5).

Table 5 - Resume table of cell behavior during exposure to the different classes of chemicals (G=Growth, NG=No growth).

Cells	Solvent	Solvent + ½ MIC LEVO	Solvent + ½ MIC TEICO	Solvent + ½ MIC THIO	Solvent + ½ MIC OME
Non-adapted cells in 1% MTBE	G. *lag* 30 hours	G. *lag* 70 hours	G. no *lag*	G. *lag* 54 hours	G. *lag* 70 hours
Non-adapted cells in 5% ethanol	G. *lag* 54 hours	N.G.	G. *lag* 30 hours	N.G.	G. *lag* 70 hours
MTBE-adapted cells	G. no *lag*	G. *lag* 20 hours	G. *lag* 41 hours	G. *lag* 41 hours	G. no *lag*
Ethanol-adapted cells	G. *lag* 52 hours	G. *lag* 52 hours	N.G.	N.G.	N.G.

When comparing non-adapted cells in the presence of MTBE and MTBE-adapted cells in the presence of MTBE, the ones that were previous adapted grew faster in the presence of 1% MTBE and in the presence of 1% MTBE+OME. FA profile was quite similar in both 1% MTBE and 1% MTBE+OME, which can be an indicator of another mechanism behind the tolerance towards omeprazole. These cells seemed to be very well adapted to the presence of MTBE, since during adaptation to these conditions cells were able to activate the suitable tolerance mechanisms. Alkene oxidizers such as *Mycobacterium* were previously shown to be able to carry out the sulfoxidation of omeprazole (Holt, Lindberg, Reeve, & Taylor, 1998). The previous adaptation to MTBE might have triggered the activation of the bacterial enzymes in order to degrade the toxic compound, increasing their biodegradability potential, which could explain the increase of the MIC value for this EPI in solvent-adapted cells. The same rational can be applied to ABC-type efflux pumps, since in the presence of thioridazine a lower lag phase in MTBE-adapted cells was observed. Nonetheless, no evidence was found in the literature that could sustain a possible biodegradation of phenotiazines by mycobacteria, which could explain the high MIC obtained for solvent-adapted cells. As for ethanol-

adapted cells, almost no growth occurred in the presence of the EPIs and the presence of cell aggregation was clearly observed. In non-adapted cells, in the presence of 5% ethanol, no growth occurred in the presence of THIO, but it was identified in the presence of OME.

In the case of antibiotics, it was shown that LEVO seemed to have no effect in the growth of *M. vaccae* previously adapted to ethanol. In non-adapted cells in the presence of LEVO, almost no growth occurred, both in 1% MTBE and 5% ethanol. This showed that the mechanisms by which *M. vaccae* adapt and overcome to the toxicity of ethanol also led to an increased resistance towards LEVO. Regarding teicoplanin, in non-adapted cells, growth was almost immediate. MTBE-adapted cells were more sensitive to TEICO than non-adapted cells. This was not expected, since according to the MIC, ethanol-adapted cells presented a higher degree of susceptibility towards these antibiotics when compared with MTBE-adapted cells.

4. Conclusions and Future Work

During exposure to different types of organic solvents, there was a dose-dependent inhibition of the cellular growth. The most toxic organic solvents were toluene, followed by MTBE, since concentrations as low as 0.1% and 2.5%, respectively, caused a very well demarked growth inhibition. When compared with ethanol and glycerol, concentrations up to 5% and 20%, respectively, were sustained. After 25 hours of growth, alterations in the fatty acid composition of cells were observed. The major difference relies in the production of 16:1 w6c FA. The higher the concentration of solvent used, the sooner the production of this FA was inhibited, with exception for glycerol, where it reached similar values when compared with control conditions (approximately 8-9% of the total FA). This trend was particularly observed in the most toxic solvents tested. As an overall response, cells increased the saturation degree in the presence of toluene, MTBE, ethanol and glycerol. This leads to the conclusion that in the presence of the organic solvents tested, cells adjusted their fluidity, by decreasing it, thus leading to more rigid membranes. It was also shown that *M. vaccae* ATCC 15483 can use ethanol as sole carbon and energy source, and in a lesser extent can also use glycerol and MTBE.

During cellular adaptation to ethanol and MTBE, cells were able to change their fatty acid composition, morphology and zeta potential. During adaptation to ethanol, cells had more negative surfaces and relied in the formation of huge cell aggregates or the shrinking of the size to overcome the toxicity of the solvent. In MTBE-adapted cells, the opposite was observed, since cells became more positive and organized in the form of rod-shaped cells, together with a lower amount of aggregation. A higher viability was achieved in ethanol-adapted cells than MTBE-adapted cells. MTBE-adapted produced BSFA during cell adaptation, and increased the saturation degree, whilst in the case of ethanol-adapted cells, the saturation degree did not exhibit such a linear relationship and no BSFA were produced.

MIC determination showed that solvent-adapted cells do not present improved MICs for antibiotics such as levofloxacin and teicoplanin, but in the case of EPIs, the MIC doubled for omeprazole and

increased 4-fold for thioridazine, in comparison to non-adapted cells. When both types of cells were re-grown in the presence of ½ MIC of each compound, the possible involvement of efflux pumps in solvent tolerance were found. The formation of lipid vesicles in MTBE-adapted cells during growth in 1% MTBE was also observed, together with EPS production in some MTBE-derived samples. Interestingly, it was shown that in non-adapted cells, the presence of teicoplanin induces the formation of cyclopropyl FA.

As future work, it would be interesting to perform this study in other classes of solvents, and use more techniques to better characterize the alterations at the level of the cell wall and membrane composition, such as changes in phospholipids head groups and mycolic acids, and physicochemical properties of cell surface. The use of techniques such as TEM would be very informative regarding morphological alterations. Other lines of work could be the complementation of this study with *in silico* analysis, particularly of metabolic pathways, in order to see if the cells possess the enzymatic machinery to biodegrade the organic solvents and to what extent. Other classes of antibiotics should be included, in order to understand if the non-acquisition of cross-resistance can be validated for other relevant anti-mycobacterial drugs. The identification of the EPS produced by MTBE-adapted cells would be other valuable information to complete this work, together with the molecular identification and characterization of the efflux pumps used by *M. vaccae* to overcome solvent toxicity.

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