Cultivation of Mycobacteria in Submerged Fermentation: Towards a Reproducible Method for Vaccine Production

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ABSTRACT

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* that to this day continues to affect millions of people. The only vaccine available to prevent the development of the disease is the Bacille Calmette-Guérin (BCG) vaccine, which is made from a live attenuated *M. bovis*, the causing agent of bovine TB. However, BCG has ranging efficacies, with one of the reasons being the cell aggregates formed, resulting in a defective quantification and quality control of the vaccine. In this study, the aggregative phenotype was studied to find conditions allowing smaller cell clusters and to obtain cells in planktonic state, using *M. smegmatis* as model organism. To this end, the effect of culture media composition and growth temperature were evaluated. Cells exposed to either 0.25% (v/v) ethanol, methanol, or DMSO presented a decrease in aggregation velocity, meaning that the cell aggregates formed were smaller. Cell aggregation also decreased after seven 15-min temperature shocks without agitation were executed. Moreover, carbon starvation led to a decrease in the size of the cell aggregates. The effect of nitrogen supplementation of the culture medium via ammonium-containing compounds in cell aggregation was studied, with cells in planktonic state being obtained during supplementation with 50 mM ammonium acetate, 50 mM ammonium chloride, or 100 mM ammonium sulphate. The fatty acid composition of the phospholipids of the membrane was assessed for every experiment, with mycobacteria responding accordingly, either by increasing or decreasing the fluidity of the membrane. Surprisingly, increased fluidity was obtained through the introduction of polyunsaturated fatty acids in the cellular membrane.

Keywords: Tuberculosis; BCG vaccine; cell aggregation; *Mycobacterium smegmatis*; culture media composition

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, and was considered by the WHO as the leading cause of death by a single infectious agent in 2020 [1]. In fact, in 2019 it was estimated that 10 million people contracted the disease and 1.4 million deaths were registered [1]. Unfortunately, the unequal access to adequate health care throughout the world, coupled with lack of treatment adherence has led to the appearance of multidrug and extensively drug-resistance strains, MDR and XDR, respectively, which require more extensive treatments with the success rate being drastically reduced [2]. Therefore, to prevent the spread of resistant strains, effective vaccination is of extreme importance. The BCG vaccine, which derives from a live attenuated strain of *M. bovis*, is the only approved vaccine for the prevention of tuberculosis [3]. However, its ability to protect adults from pulmonary TB ranges from 0% to 80%, with one of the main reasons being the lack of production standardization [4]. In turn, the absence of standardization in the production phase is mostly due to the formation of aggregates, which then leads to nonviable portions of the harvested bacteria, rendering the quality control of the vaccine difficult [4].

M. tuberculosis and other non-tuberculous mycobacteria have the ability to form aggregates in liquid cultures [5]. The hydrophobicity of the cell surface is known to have great importance in cell aggregation due to the presence of mycolic acids in the cell wall [6]. It has been found that *M. smegmatis* can regulate the chain length of mycolic acids in response to environmental factors [7].

Nevertheless, the cellular membrane can play an important role in cell aggregation. All prokaryotes can modulate the properties of the membrane, namely in its lipid composition, in response to changes in environmental conditions in order to maintain membrane fluidity [8]. This process is called “homeoviscous adaptation” [9]. In fact, the fatty acid composition can be changed through a plethora of strategies that include alterations in the chain length, degree of saturation, *cis-trans* isomerization, *iso*- or *anteiso*- branching, and cyclopropanation [8]. Mechanisms to do so consist of a *de novo* synthesis of the fatty acids of the membrane or through the modification of the fatty acid chains [8]. Increased content of saturated fatty acids lessen the membrane fluidity, while increased content of unsaturated fatty acids do the opposite, given that the former present higher melting points than the latter [8]. Moreover, the fatty acyl chain lengths also influence the membrane fluidity, with longer chains decreasing fluidity and shorter chains leading to its increase [8]. Another mechanism used to maintain membrane fluidity across different temperatures is the methyl branching of fatty acids in the *iso*- and *anteiso*- positions, with the methyl group being positioned in the penultimate or antepenultimate carbon, respectively [10].

The present study aimed at decreasing cell aggregation through the improvement of culture media conditions, such as temperature and media composition. The changes in the fatty acid composition of the phospholipids of the cellular membrane in response to the different conditions tested were assessed.

MATERIALS AND METHODS

Microorganism and growth conditions: *Mycobacterium smegmatis* ATCC 10143 was grown in 20 mL BBL™ Trypticase™ Soy Broth (TSB) from BD (France) in 100 mL Erlenmeyer flasks at 30°C and 180 rpm in an Agitorb 200 incubator (Aralab). Growth was monitored through optical density measurements at 600 nm ($OD_{600\ nm}$).

Solvent exposure: To study the effect of different solvents on cell aggregation, methanol (Fisher Scientific), ethanol (Panreac), or DMSO (Fisher Bioreagents, Fisher Scientific) were added at a concentration of 0.25% (v/v) to TSB medium, and the cell suspension was added so that the initial $OD_{600\ nm}$ was between 0.1-0.2. The cultures were grown for 24 h at 30°C and 180 rpm. The experiment was done in duplicate.

Cold shocks: To study the response to different stresses, a cell suspension was added to 20 mL of TSB supplemented with 0.25% (v/v) DMSO to obtain an initial $OD_{600\ nm}$ between 0.8-0.9. Cell cultures were grown in 45- or 55-min intervals at 30ºC and 180 rpm, followed by a 15- or 5-min stress, respectively. The stress applied was a cold shock (0ºC) without agitation (TS-NS). During the first 8 h of growth, the cell cultures suffered 5 stress shocks, with another 2 shocks being applied after 22 h of growth. The experiment was done in duplicate.

Carbon-starvation: The effect of carbon-depleted conditions in the extent of cell aggregation was studied as follows: briefly, 3-day old cultures as well as cultures harvested during exponential phase ($OD_{600\ nm}$ between 2.2-2.3) were centrifuged at 10405 *g* for 5 min (Centrifuge 5810R, Eppendorf). The supernatant of the exponential growing cells was discarded and replaced with the supernatant from the old cells and incubated at 30°C and 180 rpm for 16 h in an Agitorb 200 incubator (Aralab). The experiment was done in duplicate. Samples for the fatty acid composition analysis and zeta potential were taken before the exchange of the medium, immediately after, 1 h, and 16 h later. After 16 h, samples for microscopy were also taken

Ammonium-containing compounds as nitrogen source supplements: Nitrogen source supplementation was prepared based on a previous study, with some modifications [7]. Briefly, sterilized solutions of ammonium acetate (Sigma-Aldrich), ammonium chloride (Merck), ammonium nitrate (Riedel-de Haën), ammonium sulphate (Panreac), or ammonium bicarbonate (Sigma-Aldrich) were added at a concentration of 50, 75, or 100 mM to TSB, up to a total volume of 20 mL. Cell suspension was added to obtain an initial $OD_{600\ nm}$ between 0.1-0.2. At the 24^{th} h of growth, samples were taken for fatty acid composition and zeta potential analysis. The same procedure was done at the 42^{nd} h, with the cells being also observed by fluorescence microscopy.

Cell aggregation assay: The extent of cell aggregation was assessed by the following assay: briefly, after 24 h of growth, 16 mL of each culture were divided into four test tubes and the

$OD_{600\ nm}$ was measured over time, under static conditions. The $OD_{600\ nm}$ values decrease over time due to the sedimentation of aggregated cells, and so the percentage of cells in suspension is given by the quotient between the $OD_{600\ nm}$ at each time point and the initial $OD_{600\ nm}$. The velocity of aggregation is thus estimated by the slope of the linear regression of the percentage of cells in suspension over time during the sedimentation. When all the aggregates have been deposited, the $OD_{600\ nm}$ remains constant. After said stabilization, it is possible to distinguish up to three different cell populations, which are characterized below (Table 1). The time at which the cells stop depositing is given by the interception between the linear regression of the decreasing percentage of cells in suspension and of the constant values over time after the stabilization. The linear regression is of the following type:

$$y = mx + b \quad (1)$$

Table 1 – Cell populations obtained after the cell aggregation assay.

Cell population	Description
Mix	All types of cells that compose the culture
Top	Light aggregates at the air-liquid interface
Middle	Cells that remain in suspension
Bottom	Heavy aggregates at the bottom

Fatty acid analysis: Samples of 1 mL of cell suspension were collected and centrifuged at 8161 *g* for 2 min (µSpeedFuge SFA12K, Savant Instruments, Inc) and washed at least once with milli-Q water. The fatty acids of the phospholipids of the cellular membrane were extracted and methylated to fatty acid methyl esters (FAMEs) with the instant-FAME method from MIDI, Inc, as previously described [11]. The FAMEs were analysed by gas chromatography using a 6890N gas chromatograph (Agilent Technologies), with a flame ionization detector, an automatic injector 7683B, and an Agilent J&W Ultra 2 capillary column. Each FAME was identified using the PLFAD1 method of the Sherlock software (MIDI, Inc). The saturation degree was defined as the ratio between the sum of all the percentages of saturated fatty acids and the sum of the percentages of monounsaturated fatty acids. The unsaturation index was defined as the sum of the percentages of unsaturated fatty acids multiplied by their respective number of double bonds and divided by 100, as previously described [12].

Zeta potential: 1 mL of cell suspension in each population was collected, centrifuged at 8161 *g* for 2 min and washed at least 2 times with milli-Q water. The washed cells were then resuspended in a 10 mM KNO_3 solution and 40 µL of this suspension was then diluted in 2 mL of 10 mM KNO_3. From the electrophoretic mobility of the mycobacterial cells at 25 ^{o}C, the zeta potential was determined, according to the method of Helmholtz-Smoluchowski, by the Zeta Software version 7.11 (Malvern Instruments Ltd.) using a Zetasizer Nano ZS (Malvern Instruments Ltd.).

Fluorescence microscopy: To 1 mL of cell suspension collected from each culture in carbon-starvation conditions, 1 µL of Rhodamin B (C.I. 45170 for microscopy from Carl Roth GmbH+Co, Germany) was added. The samples were observed by fluorescence microscopy using an Olympus CX40 microscope, with an Olympus U-RFL-T burner and an U-MWG mirror cube unit (excitation filter: BP510-550; barrier filter: BA590). Images were captured by an Evolution™ MP5.1 CCD color camera using the software Image-Pro Plus, both from Media Cybernetics, Inc. (USA). Intracellular storage lipids of cells with nitrogen source supplementation were stained using Nile Red (Molecular Probes, Life Technologies, USA) as previously described [13]. The samples were observed by fluorescence microscopy as mentioned. Image analysis was performed using ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

RESULTS

Growth and aggregation of cells exposed to solvents: To assess the effect of solvent exposure in cell aggregation dispersal, *M. smegmatis* cells were exposed to 0.25% (v/v) of either ethanol, methanol, or DMSO for 24 h. Afterwards, a cell aggregation assay was performed, and the three cell populations that resulted from it were collected and the fatty acid composition of the phospholipids of the cellular membrane, biomass dry weight and zeta potential were analysed. In the assay, the $OD_{600\ nm}$ decrease over time was measured. A slower decrease associated with fewer cell aggregates formation (Figure 1A). The time at which a stabilization occurred was also determined. Therefore, the velocity of aggregation was given by the slope m, while the time of stabilization was given by the *x* value where both equations intercept (Equation 1). Both parameters obtained are summarized in the table below (Table 2).

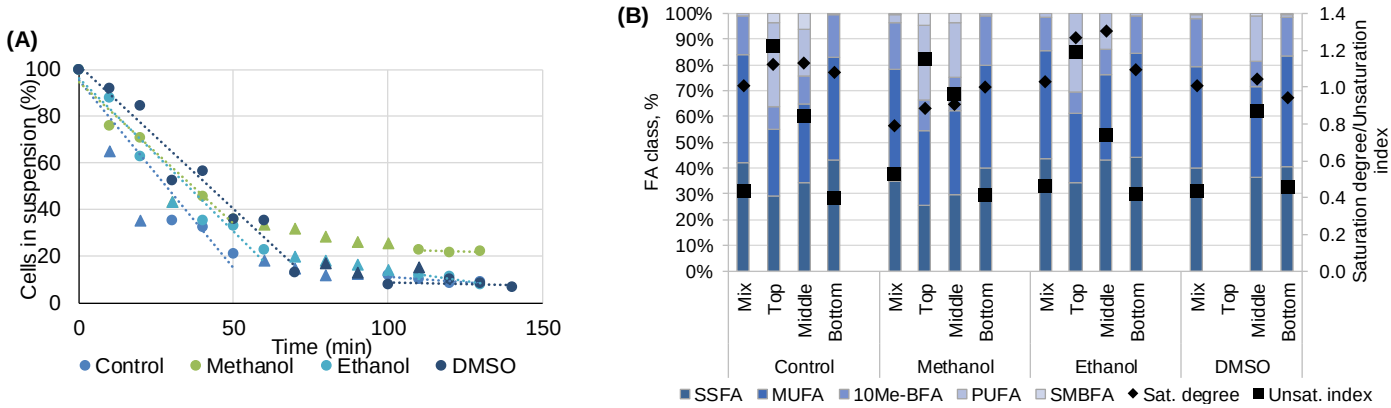

Figure 1 – Percentage of cells in suspension (A) and the fatty acid classes of the phospholipids of the membrane, saturation degree and unsaturation index (B) of cells grown on TSB alone or exposed for 24 h to 0.25% (v/v) of methanol, ethanol, or DMSO. Values considered for the equations of aggregation and stabilization (closed dots); outliers (closed triangles); linear regression (dotted lines). SSFA – saturated straight fatty acid; MUFA – monounsaturated fatty acid; 10Me-BFA – 10-methyl branched fatty acid; PUFA – polyunsaturated fatty acid; SMBFA – saturated mono-branched fatty acid.

Table 2 – Parameters obtained in the linear equations for the period of sedimentation of aggregates and stabilization for the different solvents tested.

	Aggregation equation parameters	Stabilization equation parameters	Stabilization time (min)
	m (%cells in suspension/min)	m (%cells in suspension/min)	
Control	-1.6086	-0.0952	49.29
Methanol	-1.2127	-0.0344	57.95
Ethanol	-1.3125	-0.1896	56.23
DMSO	-1.2282	-0.0278	75.06

Regarding the velocity of aggregation, when compared to control conditions, exposure to the three solvents resulted in its decrease (Figure 1A; Table 2). The stabilization time is the period at which the $OD_{600\ nm}$ values stop decreasing and only free cells or very small aggregates remain in suspension, being longer for the three conditions tested. Therefore, the smaller the cell aggregates, the longer it takes for them to deposit, and so a less cell aggregation extent is observed. Cells exposed to DMSO presented the lowest velocity of aggregation and the longer stabilization time. Moreover, cells exposed to either methanol or ethanol formed Top aggregates, which did not occur during exposure to DMSO.

The fatty acid composition of the phospholipids of cellular membrane was also assessed (Figure 1B). Namely, when compared to control conditions, the cellular membrane of the Mix population responded to methanol exposure by increasing the content in monounsaturated fatty acids (MUFAs) by 2% while decreasing the saturated straight fatty acids (SSFAs) by 7%. After the cell aggregation assay, the Top population presented phospholipids with an increased content in 10-Methyl branched fatty acids (10Me-BFAs) by 3.3% and in MUFAs by 3%, and a decreased content in polyunsaturated fatty acids of 3.4% and in SSFAs of 3.6%, in comparison to the Top population in control conditions. Cells in the Middle population adapted their membrane by increasing the content in PUFAs by 3.4%, in MUFAs by 2.1%, and in 10Me-BFAs (2.1%), with the Bottom population presenting a cellular membrane with a decreased content in SSFAs by 3%, and an increase in 10Me-BFAs by 2.6%, when compared to the respective cell populations in control conditions. Regarding exposure to ethanol, and in comparison with the corresponding cell populations in control conditions, the Mix population presented a cellular membrane with a 1.3% increase in the content of SSFAs, coupled with a decrease in 10Me-BFAs by 2.52%. The Top population was characterized by a cellular membrane with an increase in SSFAs by 5.1% and a decrease in PUFAs by 1.6%. In turn, cells from the Middle population presented phospholipids with an increase in SSFAs by 8.6% and in MUFAs by 2.4%, with a decrease in PUFAs of 4.3%. The Bottom population presented a cellular membrane with an increase in SSFAs and a decrease in 10Me-BFAs. By comparing with the

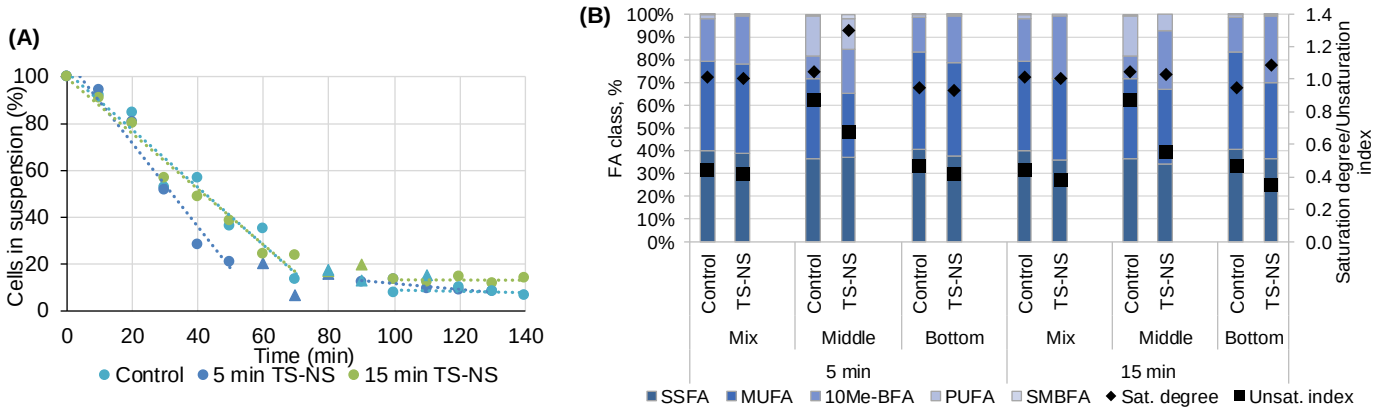

Figure 2 – Percentage of cells in suspension (A) and the fatty acid classes of the phospholipids of the membrane, saturation degree and unsaturation index (B) of cells exposed for 24 h to 0.25% (v/v) DMSO grown at 30°C (Control) or submitted to the TS-NS shocks (TS-NS). Values considered for the equations of aggregation and stabilization (closed dots); outliers (closed triangles); linear regression (dotted lines).

respective cell populations in control conditions, exposure to DMSO rendered cells from the Mix population with a decreased cellular membrane composition in SSFAs and MUFAs by 2% and 2.3%, respectively, and an increase in 10Me-BFAs of 3.4%. The Middle population presented a cellular membrane composition with increased MUFAs by 4.4% and SSFAs by 2.1%, with a concomitant 5% decrease in saturated mono-branched fatty acids (SMBFAs). The Bottom population presented a cellular membrane with an increased composition in MUFAs by 2.7%, and a reduced content of SSFAs and 10Me-BFAs by 2.8% and 1.5%, respectively. Consequently, the cells produced alterations at the cellular membrane level when exposed to methanol or DMSO that were characterized by an increase in membrane fluidity for all cell populations analysed, due to a saturation degree decrease (Figure 1B). In opposite, the response to ethanol rendered the cellular membrane more rigid, due to an increase in the saturation degree.

Cold shocks: The increase in the content of unsaturated fatty acids of the cellular membrane is closely related to the decrease in temperatures, given that the bacterial cells need to increase the fluidity of their membrane. Furthermore, the cellular membrane of cells exposed to each solvent presented an increased unsaturation index in the Middle population, when compared to the Bottom population. Therefore, to further study the effect of temperature decrease in the fatty acid composition of the phospholipids of the cellular membrane and the cell aggregation, *M. smegmatis* was exposed to 0.25% DMSO and subjected to seven cold shocks without agitation of either 5 or 15 minutes in a period of 24 h. Afterwards, a cell aggregation assay was performed (Figure 2A), and the velocity of aggregation and the stabilization time were assessed (Table 3).

Table 3 – Parameters obtained in the linear equations for the period of sedimentation of aggregates and stabilization for the thermal shocks tested.

	Aggregation equation parameters	Stabilization equation parameters	Stabilization time (min)
	m (%cells in suspension/min)	m (%cells in suspension/min)	
Control	-1.2282	-0.0278	75.06
5 min shock	-1.7786	-0.1263	50.04
15 min shock	-1.1847	-0.0041	72.51

The aggregation velocity was increased in the cells that suffered the 5-minute shocks (Figure 2A; Table 3). However, the opposite occurred with the increase in shock length, with the velocity of aggregation being reduced, when compared to control conditions. Despite the stabilization time being slightly reduced, it can be considered similar to control conditions.

Cells needed to adapt to the constant shocks applied over 24 h. At the cellular membrane-level, changes in the fatty acid composition of the phospholipids were observed

(Figure 2B). For both shocks, all cell populations presented a cellular membrane with an increase in the content of 18:0 10-methyl, and a consequent decrease in 18:1 ω9c, when compared to the control condition, with the Bottom population presenting a similar fatty acid composition of the phospholipids of the cellular membrane to the Mix population. Furthermore, cells exposed to the 15-min shocks presented a cellular membrane with decreased content in SSFAs and MUFAs in the Mix, Middle, and Bottom populations, with the PUFA content decreasing in the Middle population. Therefore, the saturation index remained constant in the Mix and Middle populations of cells submitted to the 15-minute shocks, due to the coupled decrease in SSFAs and MUFAs. Nevertheless, the saturation degree was 1.15-fold higher in the Bottom population of the challenged cells than in the corresponding population in control conditions.

Growth on carbon starvation conditions: Previous studies with *M. smegmatis* MC^2 155 have shown that carbon starvation induced a dispersion of cell aggregates [7]. Furthermore, cells resuspended in a carbon-depleted medium presented a decrease in aggregated cells. Therefore, to test whether carbon depletion would promote growth as planktonic cells, a culture of cells in exponential phase was resuspended in a 3-day old culture supernatant. Samples were taken before the exchange, immediately after, 1 h later, and 16 h later. To verify the reduction in cell aggregation in carbon-depleted conditions, cells grown on fresh TSB or on the exchanged media were observed under a microscope (Figure 3A; 3B). The culture grown on TSB for 41 h presented large cell aggregates that occupied on average 28% of the photographed area (Figure 3A). Cells resuspended on spent medium produced smaller aggregates, occupying an average area of 11% (Figure 3B).

The fatty composition of the phospholipids of the cellular membrane was assessed and as expected, before the media exchange, both conditions were equal (Figure 3C). After 1 h the compositions of the cellular membrane were still similar, given that the cells did not have enough time to adapt to the new conditions. At the 41^{st} h of growth (equivalent to the 16^{th} h of media exchange), the cells in control conditions had reached the stationary phase, and the cells with exchanged media presented a similar composition, given that they entered the stationary phase earlier due to the absence of carbon source. However, a minor increase in the content of 18:3 ω6c by 1.45% and of 16:1 ω6c by 0.85% in the phospholipids of the cellular membrane was still observed, when compared to control conditions. Therefore, the membrane of cells with exchanged media presented an increased content in PUFAs by 1.6% and in MUFAs by 0.2%, with a decrease in SSFAs of 2.1%. Also, the saturation degree and the unsaturation index of the cellular membrane were altered, with the former being 1.07-fold lower and the latter being 1.13-fold higher than the one obtained for the control condition. Alterations in the zeta potential were also observed in cells subjected to carbon starvation. The zeta potential is determined by the electrophoretic mobility of the cells, with lower values correlating with particle aggregation [14]. Until the 16^{th} h of media exchange, both conditions presented similar zeta potentials. However, at that stage, the pattern was altered, with the cells suspended in a carbon-depleted medium presenting a more negative zeta potential of -69.2 mV, similar to the one obtained 1 h after the exchange (-70.65 mV), compared to -55.2 mV of the control condition. This suggests that the cells did not need to change this parameter to adapt to carbon starvation. Cells grown in fresh medium for 41 h required alterations in their zeta potential to a lower value.

Ammonium-containing compounds as nitrogen source supplements: DePas *et al.* also found that the addition of excess ammonium, namely 75 mM ammonium chloride, to the culture medium resulted in cell aggregation being severely impaired [7]. To test this hypothesis in the present study, several ammonium-containing compounds were studied, including ammonium acetate, ammonium chloride, and ammonium sulphate. Cultures were grown

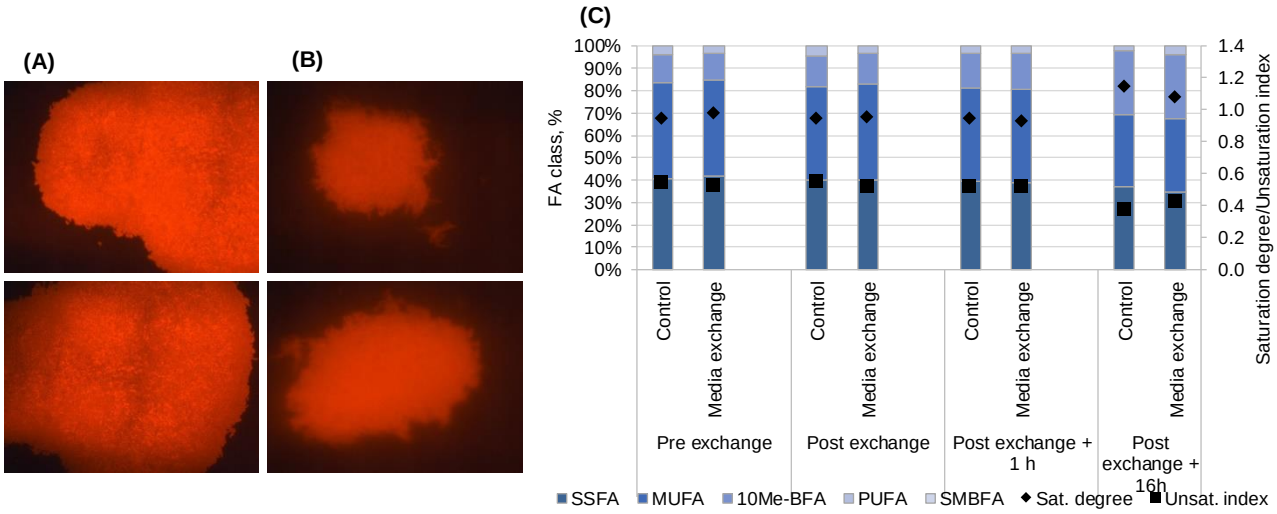

Figure 3 – Cells grown normally on TSB (A) or in carbon-depleted conditions (B) (Horizontal and vertical field widths of 8.0 µm and 6.0 µm, respectively); fatty acid classes and the corresponding saturation degree and unsaturation index of cells grown for 41 h uninterruptedly in TSB (Control) or resuspended for 16 h in carbon depleted medium pre-exchange, immediately after the exchange, 1 h, and 16 h later.

for 42 h on TSB supplemented with either 50 mM, 75 mM, or 100 mM of each compound, and samples were taken at 24 and 42 h to analyse the fatty acid composition of the phospholipids of the cellular membrane. When growth was stopped, the cells were observed by microscopy (Figure 4A), with cells in planktonic state being observed during growth with 50 mM ammonium acetate, 50 mM ammonium chloride, or 100 mM ammonium sulphate.

Despite the differences observed in the fatty acid composition of the phospholipids of the cellular membrane due to the different ammonium-containing compounds supplemented, differences were also noticed between the 24th and 42nd h of growth for all conditions (Figure 4B). Namely, the content of the cellular membrane in 10Me-BFAs increased from the 24th to the 42nd h (Figure 4B). Furthermore, with the increase in growth time, the cellular membrane was characterized by an increase in the saturation degree coupled with a decrease in the unsaturation index. However, as opposed to the remaining conditions, the cellular membrane of cells supplemented with every concentration of ammonium sulphate presented a smaller increase in saturation degree as a response to the increase in growth time. Moreover, cells supplemented with 100 mM ammonium acetate decreased the saturation degree of the membrane with the increase in growth time. After 42 h of growth, and by comparing with control conditions, cells supplemented with 50 mM ammonium acetate presented a cellular membrane with an increase in SSFAs and 10Me-BFAs and a decrease in the production of MUFAs (Figure 4). Growth supplemented with 50 mM ammonium chloride also resulted in cells with a cellular membrane with reduced composition in MUFAs, coupled with a decrease in SSFAs and an increase in 10Me-BFAs, when compared to the control. Due to the decrease in MUFAs, cells supplemented with either 50 mM ammonium acetate or 50 mM ammonium chloride presented a cellular membrane with a saturation degree 1.12- and 1.10-fold higher, respectively, than in control conditions. The cellular membrane of cells supplemented with 100 mM ammonium sulphate suffered a decrease in SSFAs and in MUFAs, and an increase in 10Me-BFAs. Therefore, the saturation degree of the cellular membrane decreased 1.03-fold, when compared to control conditions.

DISCUSSION

The present study provides useful insights into the reduction of cell aggregation of *M. smegmatis* through changes at the culture media level, e. g., different solvent

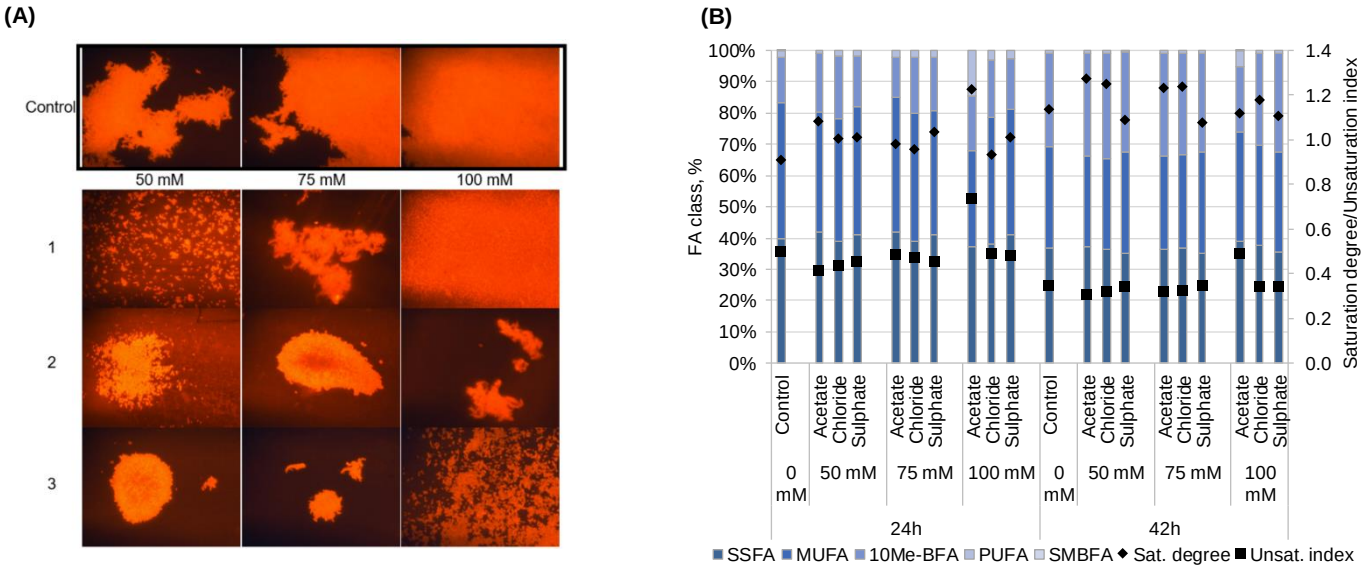

Figure 4 – Cells grown for 42 h either on TSB alone (A-Control) or supplemented with 50 mM, 75 mM, or 100 mM of ammonium acetate (A-1), ammonium chloride (A-2), or ammonium nitrate (A-3) (Horizontal and vertical field widths of 8.0 µm and 6.0 µm, respectively), and the fatty acid classes of the phospholipids of the membrane, saturation degree and unsaturation index of each ammonium-containing (B).

exposure, including DMSO, or supplementation with different concentrations of three ammonium containing compounds.

Exposure of *M. smegmatis* to 0.25% (v/v) methanol, ethanol, or DMSO led to slower sedimentation of cell aggregates, noticed by the lower absolute value of the velocity of aggregation. Moreover, the time at which all the aggregates were deposited also increased. Both results are an indication that the cells were able to reduce the size of aggregates in response to the exposure of the three mentioned solvents. Cells exposed to methanol did not reach optical densities (at 600 nm) comparable to the ones obtained during exposure to ethanol or DMSO. Nevertheless, in a previous study, *M. smegmatis* was shown to be able to grow with methanol as the sole carbon source, through the production of a methanol dehydrogenase [15]. Nevertheless, the low optical densities obtained could be attributed to a growth inhibition by methanol. The cellular membrane of cells exposed to methanol suffered a decrease in its saturation degree, which is consistent with what was previously observed in *Rhodococcus erythropolis*, given that short-chain alcohols, such as methanol and ethanol, remain in the hydrophilic portion of the phospholipid bilayer, penetrating only slightly into the hydrophobic portion [16, 17]. Therefore, to counterbalance the gaps in the membrane caused by the presence of the alcohol, cells respond by increasing the content of unsaturated fatty acids in the phospholipids of the cellular membrane [16, 18]. Furthermore, *M. vaccae* exposed to 1% ethanol also presented an increased saturation degree of the cellular membrane [19]. However, in the case of *M. smegmatis*, the cellular membrane of cells exposed to ethanol presented a higher saturation degree of the fatty acids than the cells without any solvent exposure. Exposure to DMSO rendered the best overall result, with cells presenting the slowest velocities of aggregation, coupled with a longer stabilization time. Furthermore, no cell aggregates at the air-liquid interface were observed. Given that the objective was to reduce cell aggregation, the fatty acid composition of the phospholipids of the membrane could play an important role in determining the growth as planktonic cells or cell aggregates. The phospholipids of the cellular membrane of cells exposed to DMSO presented an increase in unsaturated fatty acids when compared to non-exposed cells, with the unsaturation index consequently increasing and the saturation degree decreasing. This behaviour was

also observed in *E. coli* as a response to the presence of DMSO in the culture medium [16]. Altogether, these results show that cells exposed to either methanol, ethanol, or DMSO reduced their cell aggregation extent, and an increase in the fluidity of the cellular membrane was required during exposure to either methanol or DMSO, while cells exposed to ethanol responded through an increase in cellular membrane rigidity.

Cells in all the experiments contained a Middle population with a higher unsaturation index than the Bottom population. Moreover, the composition of the cellular membrane in unsaturated fatty acids is increased as a response to decreasing growth temperatures [8]. Growth at lower temperatures did not translate into a decrease in cell aggregation. Therefore, another approach was taken to achieve a higher percentage of cells in planktonic form, which included cold shocks and oxygen stress. After seven 15-min TS-NS shocks applied for 24 h, cells belonging to the Mix and Middle populations presented a cellular membrane with a decreased unsaturation index for all populations, which could be due to the increase observed in 18:0 10-methyl with a concomitant decrease in 18:1 ω9c. However, the saturation degree was unaltered due to the simultaneous decrease in saturated straight fatty acids. Cells from the Bottom population increased their saturation degree, with the cellular membrane becoming more rigid. The 15-min TS-NS shock returned the lowest aggregation velocity, which might mean that the extent of cell aggregation was reduced. However, the percentage of cells in suspension was decreased when compared to unchallenged cells.

It was previously reported for *M. smegmatis* that carbon starvation would lead to aggregate decrease [7]. Therefore, to test if this was applied to the strain under study, cells in exponential phase were resuspended in a 3-day old culture supernatant. The extent of cell aggregation was analysed by fluorescence microscopy, and the size of the cell aggregates was reduced. In the mentioned study, the effect on the fatty acid composition of the phospholipids of the membrane and electrophoretic mobility was not observed. However, in the present work cells responded to the carbon depletion by decreasing the saturation degree of the cellular membrane through the increase in PUFAs, thus increasing its fluidity. *R. erythropolis* DSM 1069 has also resorted to this mechanism when exposed to increasing concentrations of NaCl [20]. This adaptation mechanism could then be attributed to expressed fatty acid desaturases that modified the monounsaturated fatty acids [20].

Furthermore, cells in carbon starvation conditions presented a more negative zeta potential. While carbon-depleted medium led to a decrease in the size of aggregates, it has also been found that ammonium supplementation favours growth as planktonic cells [7]. Therefore, three ammonium-containing compounds were tested, with a decrease in cell aggregation being observed with either 50 mM ammonium acetate, ammonium chloride, or 100 mM ammonium sulphate. In fact, planktonic cells were observed. Only supplementation with ammonium chloride in cultures of *M. smegmatis* had already been tested, with 75 mM leading to accumulation of planktonic cells [7]. In the present study, the response at the membrane level was assessed, with cells increasing the rigidity of the membrane during exposure to either 50 mM ammonium acetate or ammonium chloride and rendering the membrane more fluid during exposure to 100 mM ammonium sulphate.

This study constitutes an important contribution regarding the reduction of mycobacterial cell aggregation by changing the composition of the culture media. Namely, exposure to DMSO or supplementation with ammonium-containing compounds was shown to reduce cell aggregation. Tuberculosis is still a deadly disease, especially in under-developed countries, and with mycobacterial cell aggregation being such an impairment in vaccine efficacy, the observations made in this study constitute an important step in the right direction.

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