



## Mycolic Acids and Lipids TLC Analysis of the Cell Envelope of *Mycobacterium bovis* Strains Isolated from Zacatecas, México

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### Abstract

Pathogenic Mycobacteria of the complex *Mycobacterium tuberculosis* (MTb) are the causative agents of human Tuberculosis. Current reports after COVID-19 pandemic continue to show that pathogenic mycobacteria constitutes a health public problem that is worsen by the increasing multidrug-resistance strains (MDR), and extensive drug resistance (XDR), the co-morbidities and by the lack of long term memory of the actual and official approved vaccine BCG. Approximately 1.7 million deaths annually and morbidity of 10.6 million, remaining with a latent infection, and only a 5% develop active disease. Molecular diagnostic based on real time PCR, Whole genome Sequencing, and single cell multi omics are contributing to the determination of biomarkers of the course and progression of the disease. Furthermore, several studies have highlighted that cell wall lipids of pathogenic mycobacteria play a role as a factor of virulence, and recognized by the innate immune cells, resulting in effective connection with the adaptive immune system, especially those by B and T lymphocytes. In previous work, we isolated *Mycobacterium bovis* from the bovine's nostrils in the region of Zacatecas, Mexico. Herein, we are reporting the TLC analysis of mycolic acids and lipids from the mycobacterial cell envelope. From the data, we found subtle differences in the type of mycolic acids and/or lipids profile (polar and apolar) between the *M bovis* isolates with attenuated Ha and pathogenic H37Rv *M. tuberculosis* (Hb) and *M bovis* BCG. T More detailed analysis of the natural subtle variations in composition of the cell envelope of the different geographical mycobacterial isolates is needed and deserve further exploration for potentiate the diagnostic capacity in combination with the serological and molecular techniques.

**Keywords:** COVID-19; HIV; *M. tuberculosis*

### Introduction

The infectious diseases caused by the Gram-positive bacteria, specifically of the complex of *M. tuberculosis* remains a threat in under developed countries (poor or deficient sanitary conditions or treatment and detection methods are not accessible) deficient, and in developed countries (co-morbidities) [1,2]. Tuberculosis caused from 1 to 5 million of deaths annually [1,2], while approximately 10 million individuals developed TB. It is estimated that a quarter of the world's population has or develops latent tuberculosis with *M. tuberculosis*, and of these, 3 to 10% will develop active tuberculosis [3]. The vaccine based on the attenuated *Mycobacterium bovis* Bacillus Calmette Guerin (BCG) remains the only

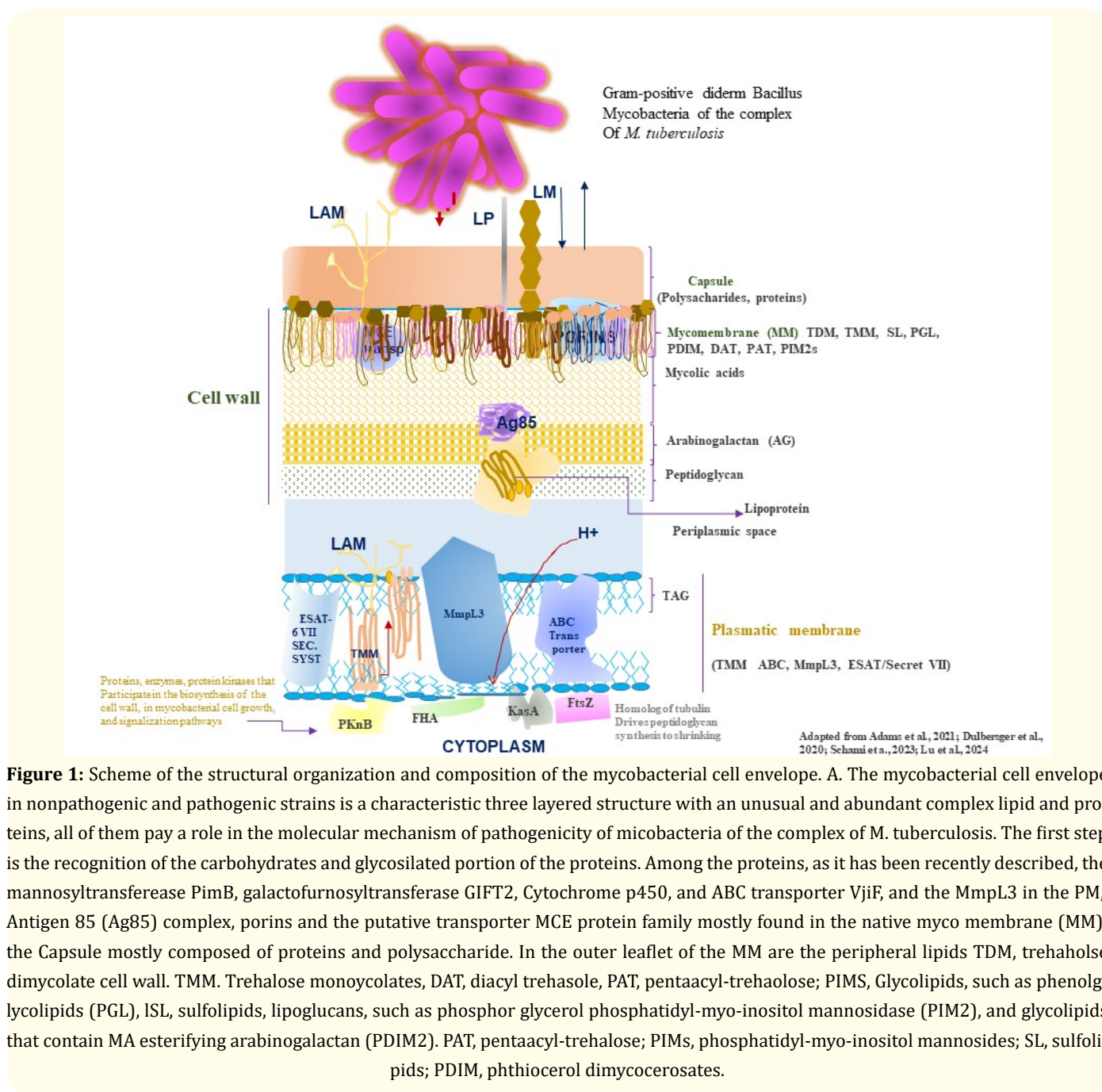
effective and officially prophylactic approved against *M. tuberculosis*. However, intense and continuous efforts for the search for other therapeutic alternatives that increase memory and protection against *M. tuberculosis* [4,5]. This scenery is worsen by the increasing rise in multidrug resistance strains, MDR, extensive resistance and super resistance strains. Thus, either improved vaccines of the BCG immunity and novel drugs that can interfere with the growth and proliferation of the mycobacterial strains [5-8]. Furthermore, co-morbidities for example with other viruses (Covid 19, HIV) or another toxin-producing bacteria, may predispose and favor *M. tuberculosis* infection [9,10]. Indeed it has been shown that natural

mutations in genes involved in the innate and/or adaptive immune response leads to genetic susceptibility to mycobacterial infections [11]. The functionality of the immune system in individual's people with mutations and epigenetic regulation is impaired upon receiving signals from the external environment that can influence the transcription and expression of genes and that play a key role in all the differentiation processes of the cells of the innate and adaptive immune system [12]. At this point as a part of the elucidation of the molecular mechanism of pathogenicity of *Mtb* much of the intense research for the dissection of the macromolecular composition of the mycobacterial cell envelope have been focused in addition to *M. tuberculosis* (canetti) in the non-pathogenic mycobacteria, *Mycobacterium. Smegmatis* (Msmg), *Mycobacterium aureus* (Mas) *Mycobacterium kansasii* (Mk), *M. avium* (Ma) *Mycobacterium gastri* (Mg) [8,13-16]. In particular, Msmg share share a homology of 61% with the *Mtb* [17,18]. But what is so particular the mycobacterial cell envelope? What is its role in terms of survival at the host cellular niche environment?. The unique cell wall of Mycobacteria. a physicochemical fortress, a strong barriers that provide strength, resistance and importantly as a mechanism for survival in the harsh environment and to circumvent the host response [19-25]. The cell envelope a dynamic and immunomodulatory structure with a complex macromolecular composition that protects enormously and effectively from the natural selection of the microbicide mechanism of the host response [12]. In particular the cell wall of mycobacteria is a three layered structure (Figure 1A-B) which elucidation and dissection has been challenging due to the different biochemical methods used for that. Despite this, current knowledge have shown that the tripartite layered mycobacterial envelope is divided grossly in the outer layered that include a surface layer, the capsule composed of polysaccharides and proteins [14,16,26-30]. The outer leaflet mycobacterial cell envelope or the so called myco membrane (MM) composed of long fatty acids, proteins (around 2000 proteins, determined by proteomics), carbohydrates bonded to lipids, and to peptides), the cell wall (enriched in addition with mycolic acids) and the inner classic plasma membrane [8,15,28-30]. The macromolecular composition makes to function to the mycobacterial cell envelope as a physicochemical barriers and shielding to the mycobacteria of the microbicide and harsh environment of the antigen presenting cells, especially of the macrophages. Furthermore the mycobacterial cell envelope serves as a path for transport and secretion of solutes and proteins that allow a proper and efficient physiology and pathogenicity, resistance as efflux pumps (transmembrane proteins) against to the different antibiotics [8,15,16,30]. Interestingly, several studies have highlighted the key role of the mycobacterial cell envelope as a dynam-

ic structure that enabled to the mycobacteria the reach and interact actively with the like receptors present in the surface membrane of the antigen presenting cells, exerting thus, immunomodulatory properties. The goal is to enter parasite the host macrophages, a very rich niche in microbicide mechanism circumvented by the evasion mechanism of *M. tuberculosis*, one of them to impair phagolysosome. [8,12,15,16,30] for long term survival. How the lipids are organized in the mycobacterial cell envelope. The di-derm Gram `positive bacteria such as tubercle bacillus more `complex than the monoderm Gram negative bacteria, which contain some 20% for the lipids, while the mycobacterial cell wall contains up to 60% of lipids. Indeed, lipids constitute 40% of the dry weight of the cell [31-33]. More recents deep insight in the definition and dissection of the mycobacterial cell envelope using biochemical tools and importantly high resolution microscopic combined with omics technologies recently shown the composition and arrangements of the mycobacterial cell envelope. The lipids comprise to one of the most features long chain fatty acids (mycolic acids, MAS), covalently linked to the cell wall polysaccharide arabinogalactan (AG) and whose esterifying trehalose [8,15,16,29,30] (Figure 1). The importance of these is utmost in terms of the development of novel and moving therapeutics targets [8,15,16,29,30]. One of the most featured components of the mycobacterial cell envelope is the lipidoarabinomannan (LAM), whose kit diagnostic developmet has improved the time of TB diagnostic, due basically to the variations in composition, organization, and location [8,15,16,29,30]. The challenge in the search for more targets for treatments in addition to diagnosis is the integration an unification of the methodologies and tools to approached because the enormous amount of strains. Of note is that molecular diagnostic based on real time PCR, whole genome sequencing have made significant improvements to the early detection, specificity sensibility. Transcriptomics have contributed also in terms of the biomarkers of the course and progression of the disease [35-37]. In previous work, we isolated *M. bovis/M tuberculosis* of human nasopharyngeal exudate, and/or from bovines nostrils. The characterization of the serological reactivity, and molecular detection systemic genic expression pattern [38-40]. Due to the fact that cell wall lipids of pathogenic mycobacteria play a role as a factor of virulence, and recognized by the innate immune cells, resulting in effective connection with the adaptive immune system, especially those by B and T lymphocytes [8,15,16,29,30,41]. Different methods have used to define and dissect the composition and organization of the mycobacterial cell envelope [8,15,16,22,29,30,42-46]. Here we are reporting a first study of the TLC analysis of mycolic acids and lipids

from *Mycobacterium bovis* isolated from bovines and compared with reference strain (Figure 2A). *M. bovis* BCG Connaught, *Mycobacterium goodnae* [22,46]. In the present work TLC analysis of mycolic acids and lipids from the mycobacterial cell envelope is investigated. From the data, we found subtle differences in the type of mycolic acids between *M bovis* isolates with attenuated Ha and pathogenic H37Rv *M. tuberculosis* (Hb) and with respect to *M bovis*

BCG. The subtle differences in the polar and apolar lipids more evident when revealed with anthrone and were between *M. tuberculosis* strains (Ha ad Hb). More detailed analysis of the natural subtle variations in composition of the cell envelope of the different geographical mycobacterial isolates (bovines, humans) is needed and deserve further exploration for potentiate the diagnostic capacity in combination with the serological and molecular techniques.



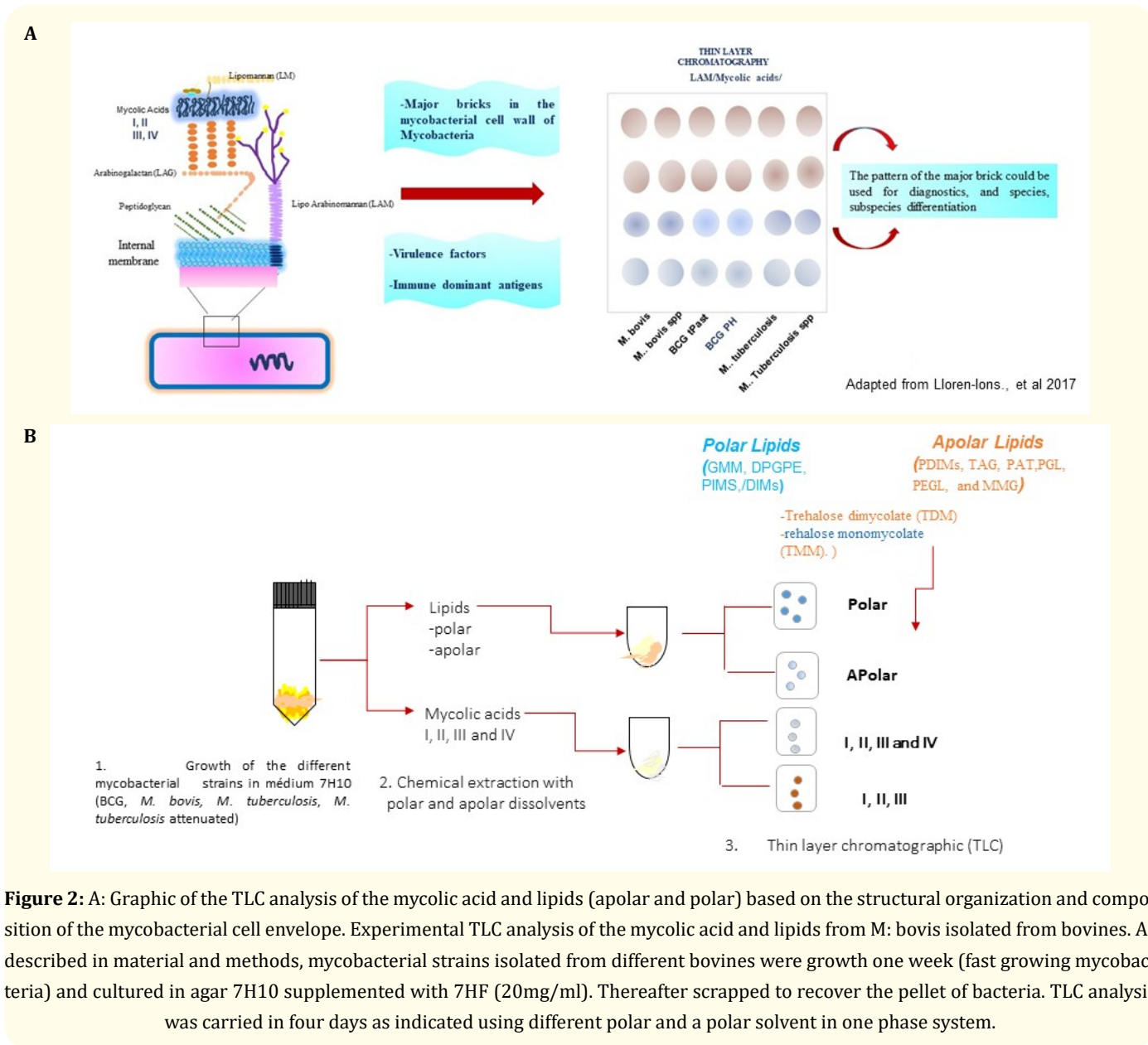
**Figure 1:** Scheme of the structural organization and composition of the mycobacterial cell envelope. A. The mycobacterial cell envelope in nonpathogenic and pathogenic strains is a characteristic three layered structure with an unusual and abundant complex lipid and proteins, all of them pay a role in the molecular mechanism of pathogenicity of micobacteria of the complex of *M. tuberculosis*. The first step is the recognition of the carbohydrates and glycosilated portion of the proteins. Among the proteins, as it has been recently described, the mannosyltransferase PimB, galactofurnosyltransferase GIFT2, Cytochrome p450, and ABC transporter VjiF, and the MmpL3 in the PM,. Antigen 85 (Ag85) complex, porins and the putative transporter MCE protein family mostly found in the native myco membrane (MM); the Capsule mostly composed of proteins and polysaccharide. In the outer leaflet of the MM are the peripheral lipids TDM, trehalose dimycolate cell wall. TMM. Trehalose monoycolates, DAT, diacyl trehasole, PAT, pentaacyl-trehaolose; PIMS, Glycolipids, such as phenolglycolipids (PGL), ISL, sulfolipids, lipoglucans, such as phosphor glycerol phosphatidyl-myo-inositol mannosidase (PIM2), and glycolipids that contain MA esterifying arabinogalactan (PDIM2). PAT, pentaacyl-trehalose; PIMs, phosphatidyl-myo-inositol mannosides; SL, sulfolipids; PDIM, phthiocerol dimycocerosates.

**Materials and Methods**

**Mycobacterial strains and growth conditions**

Mycobacteria of the complex of *Mycobacterium tuberculosis* (*M. tuberculosis*, *M. bovis*) isolated from humans and bovines, respectively, *Mycobacterium bovis* BCG (different strains) were grown in

solid medium 7H10 supplemented with 0.2% (v/v) glycerol, 0.05% (v/v) Tween 80, and enriched with 10% of OADC, at 37°C with 5% CO2 for 1 and 2 weeks. For the pathogenic mycobacteria, THF (2 mg/ml) (w/w) was added to the solid medium [40,47].



**Figure 2:** A: Graphic of the TLC analysis of the mycolic acid and lipids (apolar and polar) based on the structural organization and composition of the mycobacterial cell envelope. Experimental TLC analysis of the mycolic acid and lipids from *M. bovis* isolated from bovines. As described in material and methods, mycobacterial strains isolated from different bovines were grown one week (fast growing mycobacteria) and cultured in agar 7H10 supplemented with 7HF (20mg/ml). Thereafter scraped to recover the pellet of bacteria. TLC analysis was carried in four days as indicated using different polar and a polar solvent in one phase system.

### Thin layer chromatography (TLC)

For extraction of mycolic acids accordingly to the methodology reported [22,46] (Figure 2B). Briefly, Bacteria scraped from Middlebrook 7H9 agar plates supplemented with THF (20 mg/ml) for the case of pathogenic clinical isolate and from bovines, except in the case of attenuated *M. bovis* BCG. 50 mg of scraped bacteria to be subjected to acid Methanol lysis [22,46]. were resuspended in 2 ml of the mix of methanol, toluene and acid sulfuric acid (30:15:1), vortexed to homogenize and incubated in dry bath to 80°C for 16 h. Mycolic acids were extracted twice with n-hexane, vortexed in each time. The first and second n-hexane extracts that contained the methyl mycolates were evaporated to dryness at 40°C under nitrogen stream. The mycolates were concentrated by precipitation in cold methanol and incubated at 4°C overnight, Then, the pellet was resuspended in 50 to 100 µl in CHCl<sub>3</sub>, and analyzed by TLC on silica gel-coated plates G60 plates (20 X 20 cm) (Merck Germany). 10- 20 µl of each of the samples

were loaded to the TLC plates and developed with 100% of dichloromethane, for the mycolates II, and III and with n-hexane/diethyl ether 85:15 (v/v) for mycolates I, III and IV. The mycolates were observed as dark blue spots after the plates were sprayed with phosphomolybdic acid (VWR, United States; 10% in ethanol) and dried in the cabinet hood at room temperature. For the lipids profile determination, briefly, lipids were extracted from scraped bacteria with CHCl<sub>3</sub>:CH<sub>3</sub>OH (2:1)(v/v) with a Teflon lid, incubated at room temperature with shaking overnight. Then, organic phases (polar lipids + cellular debris) on the filter paper were dried. Scraping pellet was added to the apolar lipids dried under nitrogen (N<sub>2</sub>). This fraction in one phase extraction solvent system with CHCl<sub>3</sub>: CH<sub>3</sub>OH (1:2)(v/v) at room temperature with shaking and overnight. Then, filtered, discarding the cellular debris of the filter paper. The one phase (polar and apolar lipids) were dried under N<sub>2</sub>, and incubated for 30'-1 h with CHCl<sub>3</sub>:CH<sub>3</sub>OH and H<sub>2</sub>O with 0.1% of CaCl<sub>2</sub> and centrifuged. The recovered inorganic phase are the total extractable lipids. It washed twice with water and dried

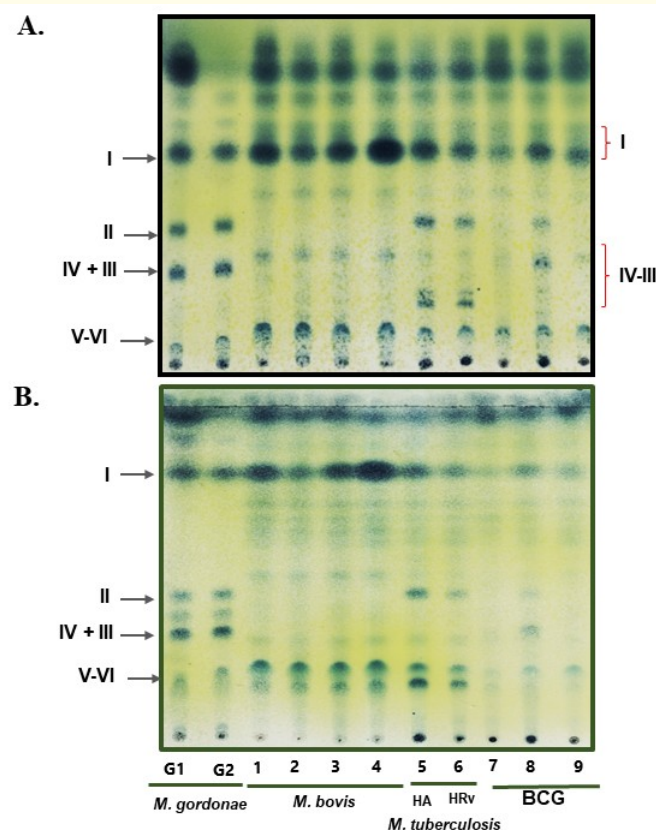
under N<sub>2</sub> before analysis. After resuspension in 100 a 200 µl) of CHCl<sub>3</sub>, the extracted lipids were analyzed by TLC, spotted (10-20 µl) onto silica gel G60 plates (20 X 20 cm), developed with the mix of CHCl<sub>3</sub>:CH<sub>3</sub>OH and methanol (96:4) for the phospholipids (PGL, PEG), with CHCl<sub>3</sub>:CH<sub>3</sub>OH:H<sub>2</sub>O (30:8:1) for the TDM, TMM, PDIM and TG [22,46]. These compounds were observed as green Turquoise and sky blue spots after the plates were sprayed with phosphomolybdic acid (VWR, United States; 10% in ethanol) and dried in the cabinet hood at room temperature.

## Results

### Mycolic acid in non- and pathogenic mycobacteria clinical and bovine isolates

Mycolic acids are components of cell wall of mycobacteria non and pathogenic bacteria serving as bricks for protection and resistance [8,15-17,22,24,29,30,46] (Figures 1, 2A). Indeed, it play a role in antimicrobial resistance to antibiotics. Here in we aimed to analyze these molecular components in mycobacteria strains isolated from humans, and compared with a reference strain and with the *M. bovis* BCG vaccine. To carry this, we performed a strategy

protocol of extraction with apolar solvents (Material and Methods) (Figure 2B), The running and separation of the mycolic acids were developed with 100% of CH<sub>2</sub>Cl<sub>2</sub> and compared with the purified mycolic acids from *M. gordonae* (LG1, G2). For all the strains analyzed (Figure 3A, lanes 1-9) the mycolic acid I were present, and it is more concentrated in pathogenic mycobacteria strains, *M. bovis* isolated from bovines (lanes 1-4), for the attenuated H37Rv and the H37Rv of *M. tuberculosis* (lanes 5-6), while for the *M. bovis* BCG (lanes 7-9) were in lower amount. The mycolic acid type II was evident in the pathogenic H37Rv with an amount similar to the reference *M. gordonae* (G1, G2). Unexpectedly one of the *M. bovis* BCG strain showed also mycolic acid type II (lane 8). Moreover, mycolic acid type III was represented in all the strains analyzed slightly higher in size than mycolic acid IV, present only in the strains *M. bovis* BCG (lanes 8-9) very low amount in comparison with the reference strain *M. gordonae* (G1, G2). Of note is that mycolic acids type III and IV differ in size between pathogenic HA and HB of *M. tuberculosis* (lanes 5-6), A similar result is with mycolic acids type V-VI, which are showed by all the strains but with a varied amount and size, especially in the analyzed *M. bovis* BCG (lanes 7-9), and the pathogenic *M. bovis* (lanes 1-4) and *M. tuberculosis* strains (lanes 5-6).

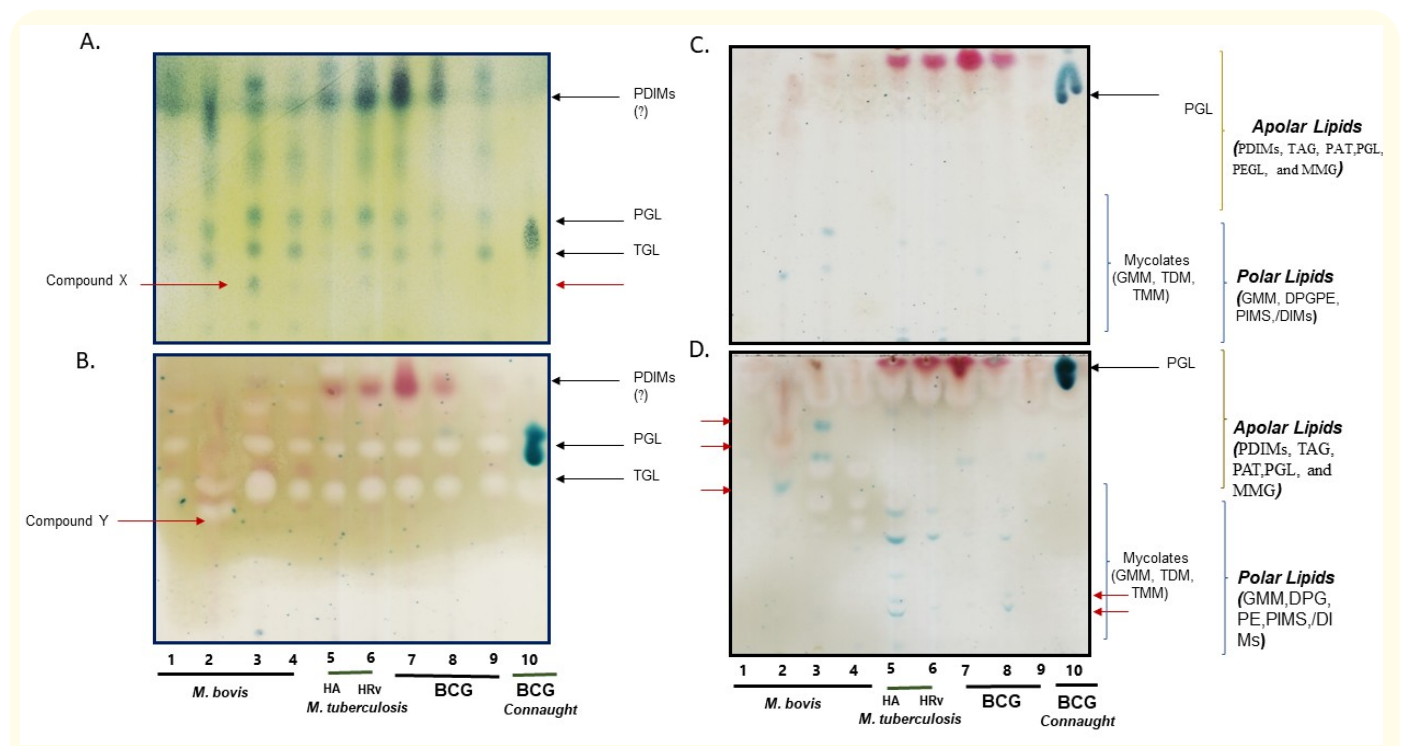


**Figure 3:** Mycolic acids analysis. A. TLC analysis of mycolic acids from pathogenic strains isolated from bovines. TLC was developed with 100% di chloromethane, after one run, mycolic acids I to VI. The reference strain of *M. gordonae* (G1, G2) B. TLC analysis of mycolic acids in hexane di-ethyl ether (85:15). *M. bovis* (lanes 1-4), *M. tuberculosis* attenuated and non-attenuated (lanes 5-6); *M. bovis* BCG (lanes 7-9).

**Lipids analysis in non-and pathogenic mycobacterial strains**

The lipids (a polar and polar) from the cell envelope of mycobacteria plays a role, structural and as immunomodulatory of the host response. In the case of the pathogenic mycobacteria have the dynamic function as virulence factors of mycobacteria, Several studies have shown the typographical, the organization and the localization of the lipids by a diverse of methods, including lipid omics combined with proteomics, with resonance magnetic nuclear (RMN) with high pressure liquid chromatography (HPLC) and Mass spectrometry, Transmission and Cryo-microscopy which have allowed to define and dissect the composition of the cell envelope [8,15-17,22,24,29,30,46]. Here we use a conventional and classic thin layer chromatography (TLC) to analyse the lipids profiles of the mycobacterial cell envelope from *Mycobacterium bovis* strains isolated from the bovines nostrils (Figures 4A-D). The TLC analysis with different proportions of polar and apolar solvents in one phase system as described in material and methods. One run with the proportion of CHCl<sub>3</sub>:CH<sub>3</sub>OH (96:4) sprayed with phosphomolibdic acid (Figure 4A) showed a similar profile of PDIMs (upper side of the plate)(lanes1-9), PGL/TGL similar even very

low amounts of compound X (red arrow) (lanes 1/6) excepte in the *M.bovis* BCG (lanes 7-9). Interestingly, with a diferent proportion of CHCl<sub>3</sub>:CH<sub>3</sub>OH (90:10) but sprayed with anthrone, a similar profile of apolar lípids was observed (Figure 4B, lanes 1-9), excepte in the compound X which was absent in all the strains analyzed. However, one *M. bovis* isolate (Figure 4B, lane 2) labeled as compound Y which was not identified. Furthermore, the apolar lípids were more evident than the polar lípids with the proportion of solvents, CHCl<sub>3</sub>:CH<sub>3</sub>OH: H<sub>2</sub>Od (60:35: 8) (Figure 4D, lanes 1-9), and increasing the amount of samples (20 ùl) loaded in the silica gel platet than with the proportion CHCl<sub>3</sub>:CH<sub>3</sub>OH: H<sub>2</sub>Od (60:16:2), and less amount of sample loaded (Figure 4C, lanes 1-9). To note is the differences (red arrows) observed in the *M. bovis* (lanes 1-3) excepte one isolate of *M. bovis* (Figure 4D, lane 4) with respect to *M. tuberculosis*, attenuated (HA)(Lane 5) and pathogenic Hb (H37Rv) (lane 6), and with respect to *M. bovis* BCG (lanes 8). Two *M. bovis* BCG strains (lanes 6 and 9) showed a diferent lípids profil-especially of the polar lípids including the mycolates, glucose mycolates (GMM), the trehalose mycolates (TMM) and trehalose dymycolates (TDM) PGL purified (Figure 4C and 4D, lane 10) from *M: bovis* Connaught.



**Figure 4:** Analysis of the total lipids extracts from the cell envelope of *Mycobacterium bovis* isolated from bovines. TLC analysis of lipids (polar and apolar) using diethyl ether (90:10 v/v), and the plates revealed with 10% phosphomolibdic acid. Lipids included trehalose dimycolate (TDM), and trehalose monomycolate (TMM). The apolar Phthiocerol dymcocersates (PDIMs), triacylglycerol (TAG), pentacyl trehalose (PAT), phenolic glycolipid (PGL) and mono-mycolyl glycerol (MMG). Polar lipids identified included glucose monomycolate (GMM), diphosphatidyl glycerol (DPG), phenylethanolamine (PE), and a range of mono- and Di-acylated phosphatidyl inositol mannoside (PIMS). PGLP, phosphoglylipids purified from *M.bovis* BCG Connaught (lane 10). *M. bovis* (lanes 1-4), *M. tuberculosis* attenuated and non-attenuated (lanes 5-6); *M. bovis* BCG (lanes 7-9). The mycobacterial cell wall is comprised of mycolic acids, Polar and Apolar lipids, Lipoarabinomanano (LAM). Either the mycolic acids or the LAM major lipid envelope.

## Discussion

In a previous work we have reported the serologic reactivity of the total extract of *M. bovis* isolated from bovines' nostrils as well as the molecular detection and systemic expression patterns of *M. bovis* infected bovines. In the present work, we are reporting the TLC analysis of the mycolic acids and lipids profile from the *M. bovis* cell envelope isolated from the bovine's nostrils. We found that there are differences especially in the type of mycolic acids (II-IV) between the *M. bovis* strains with respect to the mycolic acids showed by *Mycobacterium gordonnae* (reference strain), *Mycobacterium tuberculosis* and *M. bovis* BCG. Furthermore, there were differences in the polar lipids including the mycolates (GMM, TDM, and TMM), which were more evident with the attenuated and pathogenic strains of *M. tuberculosis* (HA and HB) and one of the *M. bovis* BCG strain analyzed.

The mycobacterial cell envelope plays a key dynamic role, due greatly to the unusual lipid composition, rich of glycolipids, glycosylated phospholipids, and complex carbohydrates with mycolic acids or peptides [20,23,24,29,46]. It has been reported that the glycolipids comprised 25% of the total dry weight. From this 40% are constituted by mycolic acids binding to trehalosa (disaccharide of  $\alpha$ -D-glucose, and formed by  $\alpha$ -D-glucopyranosyl (1-1)- $\alpha$ -glucopyranose residues) [15,16,46,48]. The glycosylated structures with O-glycosidic binding with a dual role: structural such as the polymers such as peptidoglycan and polysaccharides, and second as links of recognition with like receptor on the membranes surface of the antigen presenting cells, resulting in an immunomodulation of the host response. Moreover, these molecular fluidic glycosylated structures of the cell envelope protects from the harsh environments, and from the microbicide mechanism of the host response. Indeed, it assured the mycobacterial niche for long term survival and successful natural selection pressure, protection and Resistance [24,29,30,42,48]. Another characteristic component of the mycobacterial cell envelope that also plays a role forming a fortress called "cord factor is the trehalosa, a polymer of around 80 carbones, and it is classified in mycolates trehalose and sulpholipids of trehalose. When is acetylated is called dimycoloyl trehalosa (TDM) [24,29,30,42,48]. How these molecular composition and arrangement function and have a role in the pathogenicity mechanism of the mycobacteria of the complex of *M. tuberculosis*? [48,49], and moreover, How we can harness from this mycobacterial cell envelope composition and organization for better and precise diagnosis and treatment. First, the structural and molecular components of

the mycobacteria can act as pathogen-associated molecular patterns (PAMPs) interacting with the pattern of receptors recognition (PRRs) on antigen presenting cells [8,15,16,46]. The mannosidases residues and glycosylated -O-linked can even bind to Fc receptors and induce a strong host immune response. Indeed, the structural components of the mycobacteria have a role in the evasion mechanism that mycobacteria develop for the intracellular survival in the macrophages, and as a consequence of this, can inhibit and dampen the antimicrobial host mechanism of defense [30,46]. In terms of diagnosis and treatment, the use of many technologies have reinforced the fact that subtle differences, variations in the composition, spatial organization, represent for one side for diagnostic combined with other serologic and molecular, genetic techniques but also as a moving and promisorious target for therapeutic treatment. Based either on novel drug development or vaccine candidates. From our preliminary data we observed some differences in the pattern of mycolic acids (Figure 3A-B, lanes 1-4) extracted from these isolates and in comparison with the reference *Mycobacteria*, *M.gordonnae* (Figure 3A-B, G1, and G2). Furthermore, the lipids TLC analysis suggest differences in the amount of the unidentified compound X in one of the *M. bovis* isolates (Figure 4A, lane 3) (red arrow) with respect to the

*M. tuberculosis* (lanes 5-6) and/or *M. bovis* BCG (lanes 7-9) revealed with phosphomolybdic acid (Figure 4A). While when revealed with anthrone, an unidentified compound Y (Figure 4B, lane 2) was found. Interestingly, polar and apolar lipids were more evident and also with subtle differences between mycobacterial strains analyzed. Thus, differences in the profile of lipids especially of the mycolates (GMM, TDM, and TMM) (Figure 4D, lanes 1-4) with respect to the human pathogenic *M. tuberculosis* (Figure 4D, lanes 5-6), and unexpectedly to one of the *M. bovis* BCG (Figure 4D, lane 8). In addition, other unidentified compounds (red arrows) apolar and polar were observed especially from the *M. bovis* isolates (Figure 4D, lane 1-3), as well as in two of the *M. bovis* BCG strains (Figure 4D, lanes 7 and 9). All these differences in the type of mycolic acids and lipids profile are the current subject of detailed and further investigation. At this point it is noteworthy to mention that despite homology between the strains, exist subtle differences species specific, strengthening the fact that these variations in the composition, amount, organization and location can be combined with other serologic and molecular, genetic techniques to potentiate the diagnostic capacity for further therapeutic treatments [30,46]. Indeed, the role of the macromolecular composition of the cell wall has

been approached by lipidomics, and TLC [22,30,46]. Proteomics have revealed the multi enzymatic system, a set of 48 membrane related (cell wall, plasma membrane, cell surface, integral component of plasma membrane, integral component of membrane, cell surface, cell outer membrane) which enzymatic function participate in the synthesis of the outer and inner membrane, and cell wall composition (Arabinans, peptidoglycans, shape determination) [15,29,30,50,51], and the capsule, that combined with lipids dictates the architecture and macromolecular organization of the mycobacterial cell envelope (Figure 1). Variations in TG a triacyl glycerol, diacylglycerols (DG), and monoacylglycerols (MG) can influence the resistance or susceptibility to several antibiotics. A similar landscape is for the GP, as PIMS, the lipoglycans, lipoarabinomannan and lipomannans as characteristics PAMPS with a biologically important role in the mechanism of action of bacillus and as anti/drugs resistance mechanisms [52,56].

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### Conflicts of Interest

The authors declare not conflict of interest/the author declare that they have no competing interests.

### Author's Contributions

G.G.G.M. and E.J. Conceptualization, analysis of data. G.G.G.M. writing. N.L.T. methodology, data analysis. All authors have read and approved the manuscript.

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