Elucidating the antimicrobial mechanisms of colistin sulfate on *Mycobacterium tuberculosis* using metabolomics

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Abstract

Considering the disadvantageous of first line anti-tuberculosis (TB) drugs, including poor patient adherence, drug side effects, the long treatment duration and rapidly increasing microbe resistance, alternative treatment strategies are needed. Colistin sulfate (CS), a polymyxin antibiotic considered a last-resort antibiotics for treating multidrug-resistant *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter*, has antimicrobial activity towards mycobacteria, and could serve as a possible anti-TB drug.

Using GCxGC-TOFMS metabolomics, we compared the metabolic profiles of *Mycobacterium tuberculosis* (*Mtb*) cultured in the presence and absence of CS, to elucidate the mechanisms by which this drug may exert its antimicrobial effects.

The principal component analysis of the metabolite data indicated significant variation in the underlying metabolite profiles of the groups. Those metabolites best explaining this differentiation, were acetic acid, and cell wall associated methylated and unmethylated fatty acids, and their alcohol and alkane derivatives. The elevated glucose levels, and various glyoxylate and glycerolipid metabolic intermediates, indicates an elevated flux in these metabolic pathways.

Since all the metabolites identified in the colistin treated *Mtb* indicates an increase in fatty acid synthesis and cell wall repair, it can be concluded that CS acts by disrupting the cell wall in *Mtb*, confirming a similar drug action to other organisms.

Keywords

Colistin sulfate, Mycobacterium tuberculosis, Tuberculosis, Metabolomics, Treatment, Antibiotics

1. Introduction

Tuberculosis (TB), is an infectious bacterial disease caused by the organism *Mycobacterium tuberculosis* (*Mtb*) and usually affects the lungs (Floyd, 2014). The World Health Organisation (WHO) reports TB to be one of the world's deadliest communicable diseases, resulting in the death of up to 2 million people per annum. Furthermore, TB is considered the leading cause of death among people living with HIV (World Health Organization, 2015). TB is currently treated using the 6 month "directly observed treatment short-course" (DOTS) regimen, consisting of the four first-line drugs: rifampicin, isoniazid, ethambutol and pyrazinamide (Palmer, Chan, Dieckmann, & Honek, 2012). In patients with drug-susceptible TB, this regimen reportedly has a 1 – 4 % failure rate, and 7 % of the patients with a successful treatment outcome, reportedly relapse within 24 months (Dye, et al., 2005). The WHO has additionally reported 5% of all TB cases have multidrug-resistant TB (MDR-TB) (World Health Organization, 2015), which requires treatment using second-line anti-TB drugs (Zhenkun, 2010). These second line drugs are not only more expensive, but also have severe side effects, and an even longer treatment duration (approximately 2 years) (Baths, Roy, & Sing, 2011). These complexities, in addition to the fact that current anti-TB drugs have cross-reactions and interactions with HIV-antiretroviral therapy, emphasise the need for researching and developing new anti-TB drugs or alternative therapeutic approaches.

Colistin sulfate (CS), a polymyxin antibiotic discovered in the 1940s, is a cyclic peptide with a hydrophobic tail, and was one of the first antibiotics with significant activity against gram-negative bacteria (Ortwine, Kaye, Li, & Pogue, 2015), in particular *Pseudomonas aeruginosa* (Sabuda, et al., 2008), *Acinetobacter baumannii* (Quresh, et al., 2015) and *Klebsiella pneumonia* (Poudyal, Howden, & Bell, 2008). Colistin sulfate was proposed to function by binding electrostatically to the lipopolysaccharides and phospholipids on the outer cell membrane of these gram negative bacteria, and subsequently displace the membrane cations (magnesium and calcium) from the phosphate groups of these membrane lipids, creating pores, and subsequently causing cell death (Falagas & Vardakas, 2014). Using *M. aurum,* David and Rastogi (1985), additionally indicated that colistin sulfate has an effect on the cytoplasmic membrane of mycobacteria, and indicated a resultant cell leakage in experiments using *M. avium* (Rastogi N. , Potar, Henrotte, Franck, & David, 1988), *M. aurum*, *M. xenopi* and *M. smegmatis* (Rastogi, Potar, & David, 1986), as a consequence of cell wall disruption. Considering this evidence, colistin sulfate would also be expected to have similar effects on

Mtb. Korycka-Machala et al., (2001) subsequently showed an increased cell wall permeability in *Mtb* following colistin sulfate treatment, and Van Breda (2015) and Bax et al., (2015), that colistin sulfate also allows for elevated first line TB drug uptake in *Mtb,* as a result of this. Since most of this evidence pertaining to the anti-bacterial mechanisms of colistin sulfate in mycobacteria has been done from a histological and genomics research perspective, research using other "omics" disciplines are also required to understand this drug better, and its possible application to treating TB. Metabolomics is one of the latest additions to the "omics" technologies, and defined as an unbiased identification and quantification of all metabolites present in a sample (disease or treatment related), using advanced analytical techniques, and statistical analysis and bioinformatics, to identify the most important biomarkers for describing a perturbation (Berg, Tymoczko, & Stryer, 2007). We used a two dimensional gas chromatography coupled time-of-flight mass spectrometry (GCxGC-TOF/MS) metabolomics approach, to identify those metabolite markers best differentiating *Mtb* cultured in the presence and absence of colistin sulfate, for the purpose of confirming or elucidating its mechanism of action against *Mtb*.

2. Materials and methods

2.1 Cell culture

The cell cultures were prepared in the presence and absence of colistin sulfate, as described by van Breda et al., (2015), with slight modifications. Briefly, *Mtb* H37Ra ATCC 25177 (obtained from Ampath Pathology Laboratory Support Services, Centurion, Gauteng, South Africa) was swabbed onto Middlebrook 7H10 agar (Becton Dickinson, Woodmead, Gauteng, South Africa), supplemented with 0.5% v/v glycerol (Saarchem, Krugersdorp, Gauteng, South Africa), and enriched with 10% v/v oleic acid, albumin, dextrose, catalase (OADC) (Becton Dickinson). Our reasons for selecting a H37Ra strain in this experiment, was due to the fact that the original description of the effects of polymyxins by Rastogi et al., (1986), used H37Ra, and a recent publication by Bax et al., (2015), described similar results using H37Rv as to what van Breda et al. (2015) described for H37Ra.

The stock culture was prepared after three weeks of incubation at 37 ºC, by suspending the cells in 1 x phosphate buffered saline (PBS) (Sigma Aldrich, Kempton Park, Gauteng, South Africa) containing 0.05% v/v Tween 80 (Saarchem) to a McFarland standard of 3. Aliquots of 1 mL were stored at -80 ºC in cryovials, containing 20% v/v glycerol (Saarchem). By using the TB Ag MPT64 Device (KAT Medical, Roodepoort, Gauteng, South Africa), the presence of *Mtb* was confirmed, and the purity was determined by swabbing 100 µL of culture media onto tryptic soy agar (Merck, Darmstadt, Germany) and incubating at 37 ºC for 48 h. Before experimental investigations, a cryovial of the stored aliquots was allowed to thaw to room temperature, vortexed and swabbed onto Middlebrook 7H10 agar. Plates were sealed in Ziploc bags and incubated at 37 ºC until mid-log growth was reached (approximately 10 – 14 days).

The mid-log growth culture was suspended to a McFarland standard of 1 (using Sauton media (van Breda et al., 2015)); approximately 1 x 10^7 colony-forming units (CFU)/mL. The cell suspension (195µL) was then added to each well in a 96 well microtiter plate (Eppendorf). The antimicrobials were added to final concentrations of 0 µg/mL and 32 µg/mL colistin sulfate respectively, and the plate was sealed using sterile ziploc bags, and incubated at 37 ºC for 24 hours. The mixture in each well was subsequently transferred to Eppendorf tubes up to a volume of 1 mL. The 10 samples containing 32 µg/mL colistin sulfate and 7 samples containing no colistin sulfate, were centrifuged at 10000 x g for 1 min and showed no difference in the amounts of viable CFU/mL. The supernatant was removed and pellet rinsed and resuspended in 1 x PBS (without Tween 80) and then stored at -80ºC.

In the current investigation it is important to note, that the reason Sauton media was used, is because other media, such as Middlebrook 7H9 for instance, contains the following components which antagonize the effects of polymyxins 1. BSA (forms complexes with polymyxins) (Liu, Tyo, Martinez, Petranovic, & Nielsen, 2012), 2. Mg^{2+} and Ca^{2+} (Chen & Feingold, 1972) (D'Amato, Capineri, & Marchi, 1975), and hence it was important to use media where physiological concentrations of these divalent cations can be controlled, i.e., cation-adjusted to 10-12.5mg Mg^{2+}/L and 20-25mg Ca^{2+}/L (Falagas & Kasiakou, 2005) (Landman, Georgescu, Martin, & Quale, 2008), 3. Na^{+} (Hancock & Sahl, 2006) (Ramo´ n-Garcı´a, et al., 2013), and 4. Catalase, since the latter is an antioxidant which would inhibit polymyxin induced Fenton reaction mechanisms (Sampson, et al., 2012). Furthermore, it was important to substitute glycerol with 0.2% w/v glucose, since lower than normal MICs have been previously observed for *Mtb* when glycerol was used as the only carbon source (Pethe, et al., 2010), and with 0.05% v/v Tween 80, since *Mtb* requires the fatty acids present within Tween 80 for growth (Schaefer & Lewis, 1965) (Smith, Zahnley, Pfeifer, & Goff, 1993).

2.2 Whole metabolome extraction procedure and derivatization

Prior to GCxGC-TOFMS analysis, 0.5 mg of each of the individually cultured *Mtb* sample pellets described above were weighed into an Eppendorf tube, followed by the addition of 50 µL 3-phenylbutyric acid (0.0175µg/mL) (Sigma-Aldrich (St. Louis, MO, USA)) as internal standard. Chloroform, methanol (Burdick and Jackson brands (Honeywell International Inc., Muskegon, MI, USA)) and water were added in a ratio 1:3:1, vortexed for 1 min and then placed in a vibration mill (Retsch, Haan, Germany) with a 3 mm carbide tungsten bead (Retsch) for 5 min

at 30 Hz/s. Each sample was then centrifuged for 10 min at 10 000xg and the supernatants transferred to a GC sample vial, and subsequently dried under a nitrogen stream. Each extract was derivatized using 20µL methoxyamine hydrochloride-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Sigma-Aldrich (Darmstadt, Germany)) (containing 15 mg/mL pyridine) at 50 °C for 90 min, followed by silylation using 40µL MSTFA with 1 % trimethylchlorosilane (TMCS) at 50 °C for 60 min. These extracts were then transferred to a 0.1 mL insert in a clean GC sample vial and capped, prior to GCxGC-TOFMS analysis (Meissner-Roloff, Koekemoer, Warren, & Loots, 2012).

2.3 GCxGC-TOFMS analyses

The samples (1 µL) were analysed in random sequence, using a Pegasus 4D GCxGC-TOFMS (LECO Africa (Pty) Ltd, Johannesburg, South Africa), equipped with an Agilent 7890 gas chromatograph (Agilent, Atlanta, USA), TOFMS (LECO Africa) and Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany), in a splitless ratio. The necessary quality control (QC) samples were also analyzed at regular intervals in order to correct for any batch effects and also monitor the performance of the analysis over time. A Rxi-5Sil MS primary capillary column (30 m, 0.25 µm film thickness and 250 µm internal diameter), and a Rxi-17 secondary capillary column (1.2 m, 0.25 µm film thickness and 250 µm internal diameter) where used for GC compound separation. Helium was used as a carrier gas at a flow of 1 mL/min with the injector temperature held constant at 270 °C for the entire run. The primary column temperature was set at 70 °C for 2 min, and then increased at a rate of 4 °C/min to a final temperature of 300 °C, at which it was maintained for a further 2 min. The temperature of the secondary oven was programmed at 85 °C for 2 min, then increased at a rate of 4 °C/min to final temperature of 305 °C, at which it was maintained for a further 4.5 min. The acquisition voltage of the detector was 1700 V and the filament bias -70 eV. A mass range of 50–800 m/z was used for the mass spectra, at an acquisition rate of 200 spectra/s.

2.4 Data processing, clean-up and statistics

Mass spectral deconvolution (at a signal to noise ratio of 20), peak alignment and peak identification, were done on the collected mass spectra using ChromaTOF software (version 4.32). Identical mass spectra of the compounds in each of the samples were aligned, if they displayed similar retention times. Compounds were identified by comparison of their mass

fragment patterns and retention times, to that of libraries compiled from previously injected standards.

Following the data processing steps described above, a standardized metabolomics data clean-up procedure was conducted (Smuts, Der Westhuizen, Francois, Louw, & al., 2013). Normalization of each of the detected compounds was done using the total useful MS signal (TUS) (Chen, et al., 2013) and by calculating the relative concentration of each compound, using the internal standard as a reference. A 50% filter was applied in order to remove those compounds showing more than 50% zero values within both groups (Lutz, Sweedler, & Wevers, 2013) and the QC samples used to correct for any batch effects, using quantile equating (Wang, Kuo, & Tseng, 2012). Additionally, a 50% QC coefficient of variation (CV) filter was applied (Godzien, Alonso-Herranz, Barbas, & Armitage, 2014), and all zero-values were replaced by a value determined as half of the smallest concentration (i.e. the detection limit) detected in the entire data set, as these may be due to low abundance rather than being absent (Piotr, Xu, & Goodacre, 2014).

The data were subsequently analysed using a variety of multi- and univariate statistical methods, using a web based software package supported by the Metabolomics Society: MetaboAnalyst (based on the statistical package “R”; version 2.10.0), and included principal components analysis (PCA) (Buydens, et al., 2009), partial least squares–discriminant analysis (PLS–DA) (Cho, et al., 2008), a t-test and effect size calculations (Smith, Is it the sample size of the sample as a fraction of the population that matters?, 2004).

3. Results

Figure 1 shows clear PCA differentiation between the individually cultured *Mtb* samples in the presence and absence of colistin sulfate. This natural differentiation of the samples of each of the sample groups can be ascribed to the variation in the total metabolite profiles of each, as determined by GCxGC-TOFMS. The total variance explained by the first two principal components (PCs) (R2X cum) was 48.4% of which PC1 contributed to 37.5% and PC2 10.9%, respectively.

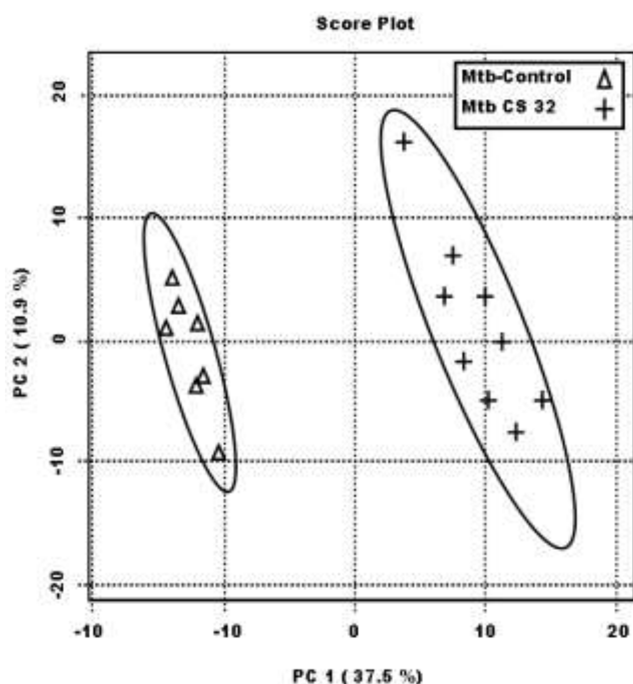

Figure 1: PCA differentiation using the GCxGC-TOFMS whole metabolome analysed data of the individually cultured Mtb in the absence (Mtb-Control) and presence (Mtb-CS) of colistin sulfate (32 mg/mL). The variances accounted for are indicated in parenthesis.

Subsequently, those metabolites that contributed most to this differentiation were selected on the basis of complying with all of the following criteria: a PCA modelling power > 0.5 (Buydens, et al., 2009), a PLS-DA VIP value > 1 (Cho, et al., 2008), a t-test P-value < 0.05 and an effect size > 0.5 (Smith, 2004).

Figure 2 is a summary of the number of metabolite markers selected by each of the univariate and multivariate statistical approaches described above, as well as the selection of the 21 metabolites listed in Table 1, considered most important for explaining the variation detected.

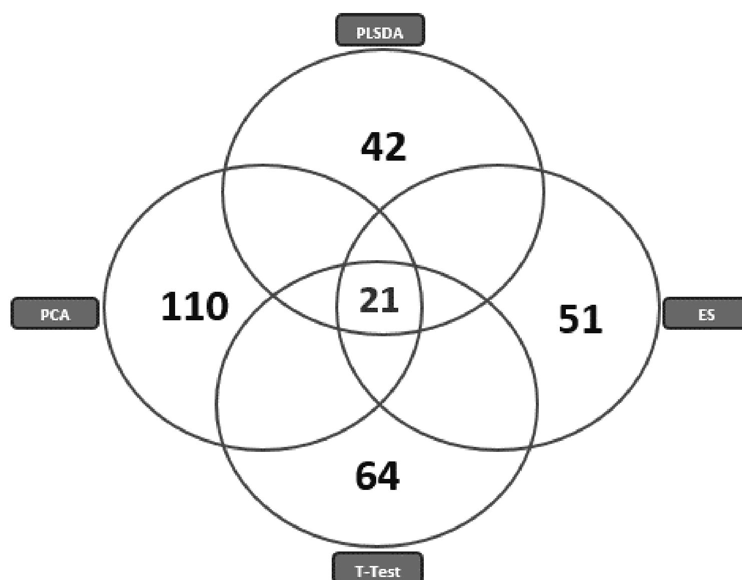

Figure 2: Venn diagram illustrating a multi-statistical approach for selecting the 21 metabolite markers best describing the variation detected between the individually cultured *Mtb* samples in the presence and absence of colistin sulfate.

4. Discussion

As previously mentioned, treatment with colistin sulfate results in a structural disruption of the cell wall in *Mtb* (Bax, et al., 2015). The metabolite markers detected in the colistin sulfate treated *Mtb* in the current metabolomics investigation confirms this, and additionally indicates that *Mtb* attempts to rectify this by upregulation of its fatty acid synthesis pathways for subsequent cell wall repair. Accompanying this is an upregulation of glycolysis. The mechanisms associated with this, in the context of the metabolite markers detected, will be described in detail below and are summarized in Figure 3.

In Table 1, 15 out of the 21 metabolite markers identified by the statistics described above, are directly linked to elevated fatty acid biosynthesis and subsequently also cell wall synthesis. These included hexadecanoic acid, octadecenoic acid, eicosanoic acid and hexacosanoic acid, all of which are known to form methyl-branched chain fatty acids and ultimately the mycolic acids (Du Preez & Loots, 2012), an important component of arabinogalactan (AG) in the cell wall core of *Mtb* (Kaur, Guerin, Škovierová, Brennan, & Jackson, 2009). Additionally, although not detected as part of the 21 metabolite markers, decanoic acid (0.100 vs. 0.219 µg/ml; $P < 0.05$), dodecanoic acid (0.421 vs. 0.592 µg/ml; $P < 0.05$) and octadecanoic acid (0.491 vs.

Table 1: The 21 metabolite markers that best explain the variance between the individually cultured *Mtb* samples in the absence (*Mtb*-Controls) and presence (*Mtb*-CS) of colistin sulfate.

Metabolite name (Chemspider ID)	*Mtb* controls: Average concentration (mg/g cells) (standard error of the mean)		Mtb treated with colistin sulfate Average concentration (mg/g cells) (standard error of the mean)		PCA (Power)	PLS-DA (VIP)	Effect sizes (*d*-value)	*t*-test (*P*-value)
Octanoic acid (NSC 5024 [DBID])	0.205	(0.192)	0.019	(0.010)	0.787	1.423	0.966	>0.001
Hexadecanoic acid (NSC 5030 [DBID])	0.860	(0.176)	1.259	(0.198)	0.948	1.224	2.022	0.001
Octadecenoic acid (NSC931 [DBID])	0	(0)	0.021	(0.005)	0.861	1.165	4.580	0.003
Eicosenoic acid (4445895)	0	(0)	0.006	(0.007)	0.842	1.181	0.930	0.002
Hexacosanoic acid (NSC 4205 [DBID])	0	(0)	0.059	(0.038)	0.804	1.244	1.541	0.001
Methyldecanoic acid (LMFA01020090)	0.013	(0.004)	0.017	(0.004)	0.822	1.238	0.994	0.001
Methyldodecanoic acid (4445769)	0.045	(0.011)	0.115	(0.025)	0.855	1.123	2.794	0.004
Methyltetradecanoic acid (NSC 189699 [DBID])	0.317	(0.021)	0.789	(0.261)	0.893	1.073	1.810	0.007
Sebacic acid (NSC 19492 [DBID])	0.001	(0.001)	0.004	(0.003)	0.898	1.151	1.278	0.003
Tetradecanol (NSC 4194 [DBID])	0.016	(0.003)	0.059	(0.019)	0.982	1.516	2.251	>0.001
2-Ethyl-2-Methyl-Tridecanol (921600091)	0.006	(0.001)	0.044	(0.033)	0.809	1.245	1.159	0.001
5-Nonanol (NSC4552 [DBID])	0.001	(0.001)	0.023	(0.004)	0.965	1.054	6.168	0.001
Hexadecane (NSC 172781 [DBID])	0.002	(0.003)	0.031	(0.029)	0.978	1.555	1.024	>0.001
Octadecane (NSC 172781 [DBID])	0.020	(0.011)	0.115	(0.028)	0.878	1.197	3.446	0.002
Octacosane (NSC 5549 [DBID])	0	(0)	0.016	(0.016)	0.993	1.611	0.979	>0.001
Methyltetradecane (NSC 172781 [DBID])	0.005	(0.004)	0.044	(0.012)	0.664	1.090	3.186	0.006
Glucose (NSC4552 [DBID])	0.195	(0.187)	0.366	(0.170)	0.819	1.470	0.913	>0.001
Oxalate (c0017 [DBID])	0	(0)	0.006	(0.006)	0.863	1.266	0.954	0.001
Glycerol (NSC 9230 [DBID])	0.063	(0.012)	0.082	(0.011)	0.891	1.173	1.469	0.002
Monopalmitin (110006)	0.165	(0.058)	0.216	(0.056)	0.987	1.614	0.874	>0.001
Propyl myristate (AI3-31609 [DBID])	0.014	(0.003)	0.023	(0.006)	0.991	1.626	1.328	>0.001

0.888 µg/ml; $P > 0.05$) were also found to be elevated, further supporting this. Another important observation was the elevated levels of the methylated branched fatty acids (methyldecanoic acid, methyldodecanoic acid and methyltetradecanoic acid), in the colistin sulfate treated group comparatively, which in turn not only serve as substrates for mycolic acid synthesis, but also

function as hydrophobic modulators of the host's cellular immune function, and various virulence factors in the microbe (Lee, VanderVen, Fahey, & Russell, 2013). These methylated fatty acids are proposed to be formed by 3 possible routes: 1. fatty acid methylation via S-adenosylmethionine (SAM) functioning as the methyl donor (Du Preez & Loots, 2012), 2. methylmalonyl-CoA derived polyketide synthase complexes, originating from propionyl-CoA and malonyl-CoA (Duncan & Garton , 2007) and 3. acetyl-CoA metabolism to butyric acid, which in turn reacts with propionyl-CoA (Massey, Sokatch, & Conrad, 1976).

Further substantiating these results, are the presence of various alcohols (tetradecanol, nonanol and 2-ethyl-2-methyltridecanol) and alkanes (hexadecane, octadecane, octacosane and methyltetradecane) corresponding to the aforementioned fatty acids (Park, 2004). Additionally, although not detected using the marker selection process described above, decanol (0 vs. 0.018 µg/ml; $P < 0.05$), decane (0.006 vs. 0.012 µg/ml; $P > 0.05$), dodecane (0 vs. 0.007 µg/ml; $P > 0.05$), eicosane (0.083 vs. 0.172 µg/ml; $P < 0.05$), tetracosanol (0.001 vs. 0.006 µg/ml; $P > 0.05$), tetracosane (0 vs. 0.01 µg/ml; $P < 0.05$), methylhexacosane (0.132 vs. 0.611 µg/ml; $P > 0.05$), tetramethylhexadecanol (0.001 vs. 0.023 µg/ml; $P < 0.05$) and metylhexadecane (0 vs. 0.01 µg/ml; $P > 0.05$)), were also seen to occur in elevated amounts, further confirming these mechanisms.

Another important observation supporting the unanimous metabolic flux observed in this study towards fatty acid biosynthesis and cell wall repair, is the significantly elevated concentrations of glucose, glycerol and monopalmitic acid. According to de Carvalho (2010), *Mtb's* central carbon metabolism is able to co-catabolise multiple carbon sources for energy (de Carvalho, et al., 2010). Considering the colistin sulfate treated *Mtb's* need to preferably utilize fatty acids towards cell wall repair, one would expect that this organism would subsequently resort to glucose, which was freely available in the growth media, as the primary energy substrate, in conjunction with an upregulated glyoxylate cycle (Badejo, et al., 2013), substantiated in this investigation by the elevated glucose and oxalic acid detected (Coad, Friedman, & Geoffrion, 2012). Furthermore, as shown in Figure 3, various intermediates of glycolysis, can additionally serve as substrates for fatty acid biosynthesis, including acetyl-CoA, as previously mentioned, and glyceraldehyde-3-phosphate (G-3-P) via glycerol (Berg, Tymoczko, & Stryer, 2007), the latter of which is supported by elevations in monopalmitic acid and the glycerol present in the growth media.

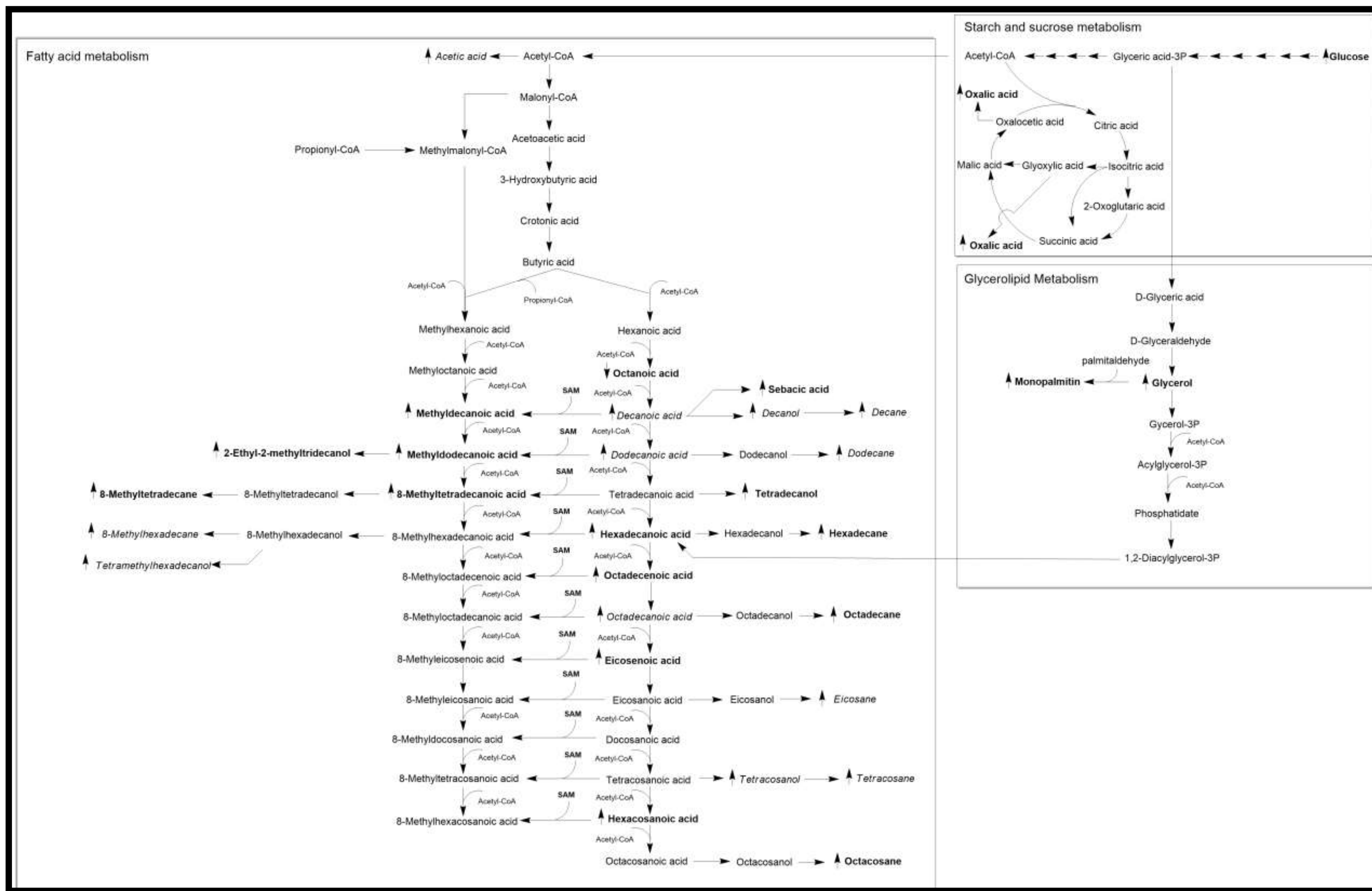

Figure 3: Altered *Mtb* metabolome induced by treatment with colistin sulfate. The schematic representation indicates the 21 metabolite markers in bold and the confirmatory metabolites which were also elevated, but not necessarily significantly so, indicated in italics. Increase and decrease in the metabolite markers are indicated by ↑↓ respectively.

5. Concluding remarks

This study, is the first of its kind to use a metabolomics research approach in order to identify biomarkers explaining the antibacterial mechanisms of colistin sulfate against *Mtb, and* additionally shows the capacity of metabolomics for identifying metabolite markers which can be used to better understand or confirm drug action. The fatty acid metabolite markers identified in the colistin sulfate treated *Mtb*, shows a metabolic flux towards fatty acid synthesis and cell wall repair. Furthermore, glucose uptake is increased, serving as the preferential energy source (as opposed to fatty acids which are now preferentially being used for cell wall repair) to fuel an upregulated glyoxylate cycle, and additionally as a precursor for further cell wall fatty acid synthesis via the glycerolipid metabolic pathway. Considering this, it can be concluded that colistin sulfate acts by disrupting the cell wall in *M. tuberculosis*, confirming a similar drug action as that seen in other organisms.

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7. Transparency declarations

None to declare.

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