

Extremophile-derived *Bacillus licheniformis* metabolites selectively remodel macrophage bioenergetics following exposure to *Mycobacterium tuberculosis*-derived metabolites

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Article

Keywords: *Bacillus licheniformis*, *Mycobacterium tuberculosis* H37Rv, mitochondrial modulation, cellular reprogramming, host-metabolite modulation, macrophage metabolism, microbial-host interactions

Posted Date: October 19th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10994809/v1>

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Additional Declarations: No competing interests reported.

Abstract

Secondary metabolites produced by *Mycobacterium tuberculosis* (*M. tuberculosis*) have been implicated in the hijacking of macrophage antibacterial responses through metabolic reprogramming that favors pathogen survival. This study investigated the potential of *Bacillus licheniformis* secondary metabolites to counteract the effects of secondary metabolites from *M. tuberculosis* H37Rv on RAW264.7 macrophages. Functional shotgun metagenomics and metabolomics (LC-MS-QTOF) were employed to tentatively identify the bacteria and their metabolic profiles. Additionally, the responses of RAW264.7 macrophage cells to bacterial secondary metabolite treatment were assessed using high-resolution respirometry (Oroboros O2k), reactive oxygen species (ROS) and nitric oxide (NO) assays, and RNA sequencing to gain mechanistic insights. The gold mine tailings were found to harbor metal-tolerant microbial communities, predominantly comprising taxa such as *Bacillus* and *Clostridium*. Tentative genomic and metabolite analyses indicated the potential of *Bacillus licheniformis* to produce surfactin, lichenysin A, and fengycin homologs. In contrast, the secondary metabolites tentatively identified as produced by *M. tuberculosis* H37Rv were glycolipid homologs. Metabolites from *M. tuberculosis* H37Rv were found to reduce routine respiration and impair the TCA cycle's contribution to OXPHOS. Conversely, subsequent treatment with *B. licheniformis* metabolites was mechanistically linked to transcriptional remodeling associated with autophagy and enhanced cellular stress management. These findings demonstrate the potential of extremophile-derived *B. licheniformis* secondary metabolites to counteract the effects of *M. tuberculosis* metabolites that are associated with metabolic stress.

Introduction

Gold mine tailings harbor intricate microbial communities that are crucial for regulating nutrient cycling, toxic metal mineralization, and stabilization [1,2]. These subterranean microbial communities have been identified as regional drivers in the rehabilitation of mine tailings, significantly influencing aboveground biotic communities, including plant species diversity and productivity [3,4]. The exploration of natural products (NPs) synthesized by bacteria is a promising avenue for discovering potent chemotherapeutics against infectious diseases such as tuberculosis (TB) [5]. A previous study reported the antimycobacterial activity of NPs derived from *Bacillus licheniformis* (*B. licheniformis*) OM182835 and *Bacillus subtilis* (*B. subtilis*) OM182840, isolated from gold mine tailings [6]. Previous investigations into the *B. subtilis* group have highlighted the antimicrobial potential of surfactin, iturin, and fengycin analogs, demonstrating their effectiveness against antimicrobial-resistant strains [7,8]. Despite the demonstrated efficacy of *B. licheniformis* bioactive secondary metabolites against *M. tuberculosis*, research on their ability to reprogram the host immunoparalysis induced by *M. tuberculosis* metabolites remains limited. Thus, it is crucial to investigate the potential of *B. licheniformis* metabolites to reprogram macrophages and counteract the detrimental effects induced by *M. tuberculosis* metabolites.

Cell-mediated immunity is integral to the defense against *M. tuberculosis* infection, encompassing mechanisms such as oxidative and inflammatory responses, autophagy, apoptosis, and antigen processing and presentation by M1 macrophages and *M. tuberculosis*-specific CD4 + T cells [9].

Concurrently, the secondary metabolites and virulence factors of *M. tuberculosis* modulate both infected and uninfected host cells at the single-cell and subcellular levels. The literature indicates that the distinct life stages of *M. tuberculosis* contribute to unique gene expression profiles in macrophages, resulting in diverse macrophage states [10]. For instance, when activated by lipopolysaccharide (LPS), M1 macrophages increase the expression of *Acod1*, the gene encoding aconitate decarboxylase, an enzyme that converts cis-aconitate to itaconate [11]. There is evidence that itaconate inhibits the Krebs cycle enzyme succinate dehydrogenase (SDH) and blocks reactive oxygen species (ROS) production from complex I [11]. Furthermore, there is a direct link between the negative regulation of mitochondria via the *Acod1*-itaconate-SDH axis and the inhibition of hypoxia-inducible factor (HIF)-1 α activity and interleukin (IL)-1 β production [11,12]. Interestingly, prolonged activation of the *Acod1*-itaconate-SDH axis during chronic inflammation induces immunoparalysis, rendering cells more vulnerable to secondary infections [13,14].

Recent studies have highlighted the critical role of mitochondrial dynamics as cellular hubs in both the innate immune response and pathways leading to host cell death [13]. Emerging evidence within the signaling paradigm suggests that mitochondria and phagolysosomes engage in integrated crosstalk that determines the cellular state and is associated with the control of bacterial infection [15]. The AMP-activated protein kinase (AMPK) pathway has been implicated in immunometabolic reprogramming in response to stress signals triggered by *M. tuberculosis* infection [16]. However, *M. tuberculosis* disrupts AMPK signaling, thereby inhibiting phagosomal maturation through virulence factors such as the phosphatase PtpA and Rv1184, which interact with the V-ATPase subunit H and prevent phagosomal acidification [17,18]. In contrast, *B. licheniformis* can counteract the immunoparalysis induced by lipopolysaccharides and pathogenic Gram-positive bacteria, including *Clostridium perfringens*, in both murine and porcine models (Yu et al., 2023; Zhong et al., 2025). Consequently, this study hypothesizes that the secondary metabolites produced by the non-pathogenic strain *B. licheniformis* OM182835, isolated from gold mine tailings in Germiston, Johannesburg, South Africa, may reprogram the immunoparalysis induced by metabolites from *M. tuberculosis* H37Rv.

Materials and methods

Sample collection and evaluation

Gold mine tailings samples were collected from Johannesburg, South Africa (26°13'7.08" S, 28°29'8.64" E, 1620 m altitude above sea level) (Fig. 1). A total of 0.5 kg of samples were collected from various locations within the area, each extracted at a depth of 12 cm, and subsequently amalgamated to form a composite sample [6]. The samples were preserved in polyethylene bags at 4°C for subsequent analysis. The initial phase of the analysis focused on characterizing key soil properties. Soil pH and electrical conductivity (EC) were measured as previously described using a glass electrode pH meter (Crison Instruments, Spain) [21]. Total carbon (TC), total nitrogen (TN), and carbon-to-nitrogen (C:N) ratio were quantified using the dry combustion method with an EuroVector Elemental Analyzer [22]. The permanganate oxidizable carbon (POXC) content was evaluated as described previously [23].

Cell lines

The Raw264.7 (ATCC TIB-71) macrophage cell line was assessed for mycoplasma contamination utilizing a Mycoplasma detection kit. The cell line was generously accessed from the Division of Molecular Biology and Human Genetics at Stellenbosch University. The cells were maintained in a humidified incubator at 37°C with 5% CO₂. *Mycobacterium tuberculosis* H37Rv stocks were preserved in 50% glycerol at – 80°C. Working stock cultures were maintained in Middlebrook 7H9 broth supplemented with 10% OADC (oleic acid albumin dextrose catalase; Becton Dickinson, USA) and incubated at 37°C.

Metagenomic sequencing, taxonomic composition, and functional profiling

Genomic DNA was extracted from soil samples and purified using the Promega Wizard® Genomic DNA Purification Kit, following the manufacturer's protocol. Furthermore, *Bacillus* spp. enrichment was conducted as previously described [6,24], with DNA extraction performed using the same purification kit. The concentration and quality of the DNA were evaluated with a microplate spectrophotometer, focusing on the 260/230 and 260/280 absorbance ratios. Metagenomic sequencing was performed on the Illumina NextSeq platform at Inqaba Biotech. Briefly, the genomic DNA was enzymatically fragmented using the NEB Ultra II FS kit, following the manufacturer's instructions. DNA fragments ranging from 200 to 500 base pairs were size selected using AMPure XP beads. Post-size selection, the fragments underwent end-repair and ligation with Illumina-specific adapter sequences. Each sample was individually indexed and subjected to a second round of size selection. Following this, the samples were quantified by fluorometry, diluted to a standard concentration of 4 nM, and sequenced on the Illumina NextSeq platform using a NextSeq 300 cycle kit, in accordance with the manufacturer's standard sequencing protocol. Each sample yielded a total data output of 3 Gb, resulting in paired-end reads of 2x150 base pairs.

Bacterial sequence data were processed as described previously [25,26]. Initially, the quality of the raw paired-end Illumina reads in (.fastq) format was assessed using FastQC (v0.11.9) [27]. To improve the quality of the raw reads, low-quality bases and adapter sequences were trimmed using Trimmomatic (v0.36) [28]. Following trimming, the reads underwent a subsequent quality check with FastQC (v0.11.9) [27]. Taxonomic profiling was conducted using Metaphlan4 (v.4.beta.2, database vJan21_CHOCOPHiAnSGB_202103) [29]. High-quality reads were assembled into contigs using MetaSPADES (v3.15.3) [30]. The assembled contigs were binned into metagenome-assembled genomes (MAGs) using MaxBin2 (v2.2.4) and MetaBAT2 (v1.7). Integration of the MAGs was achieved with the DAS tool (v1.1.2). The quality of the bins was evaluated using contamination (< 10) and completeness (> 90) metrics with CheckM (v1.0.18) [31], ensuring the selection of high-fidelity genomic bins for further analysis. The taxonomic classification of the MAGs was determined using the Genome Taxonomy Database Toolkit (GTDB-Tk) [32], and phylogenetic trees were visualized using iTOL v7 [33]. Functional

annotation of the assembled genomes was performed using the Rapid Annotations using Subsystems Technology toolkit (RASTtk) (v1.073). Additionally, HMMER (v3.2.1) was used to identify enzymes associated with environmental bioelement-cycling families. The MAGs were functionally annotated and refined using DRAM KBase (<https://www.kbase.us/>) [34]. Finally, MAGs were deposited in NCBI, and corresponding accession numbers were assigned. The biosynthetic gene clusters (BGCs) and core genes within the genome were annotated using antiSMASH v5.0.0 (available at <https://antismash.secondarymetabolites.org>) [35]. A relaxed detection strictness was implemented to optimize the identification of pathways responsible for secondary metabolite biosynthesis. The functional analysis of metal processing genes within the MAGs was annotated using MagicLamp [36].

Secondary metabolite production for *Mycobacterium tuberculosis* H37Rv

Mycobacterium tuberculosis H37Rv cultures were prepared as described previously [6] in Biosafety level 3 (BSL3). Briefly, bacterial stocks were preserved in 50% glycerol at – 80°C. Working stock cultures were cultivated in Middlebrook 7H9 broth, supplemented with 10% OADC (oleic acid albumin dextrose catalase; Becton Dickinson, USA), and incubated at 37°C. Starter cultures were adjusted to an OD₆₀₀ of 0.05 and expanded in 250 mL flasks containing Middlebrook 7H9 broth, then incubated at 37°C with continuous shaking at 200 rpm for 21 days. Secondary metabolites were subsequently prepared and sterilized by filtration through 0.2 µm filters. To confirm the absence of viable *M. tuberculosis*, 100 µl aliquots of the filtrate were plated onto Middlebrook 7H10 agar and incubated at 37°C for up to 8 weeks [37,38]. Samples that did not exhibit colony formation were classified as sterile and were then processed in BSL2 for subsequent assays. Subsequently, these sterile samples were lyophilized to facilitate storage and prepare them for metabolite extraction with solvents.

Secondary metabolite production for *Bacillus licheniformis* OM182835

The stock culture of *B. licheniformis* OM182835 was obtained from a prior study by Nyambo et al. (2024). For secondary metabolite production, a starter culture of *B. licheniformis* OM182835 was prepared by inoculating the pure isolate into 50 mL of tryptic soy broth in Erlenmeyer flasks, as described previously [6]. Tryptic soy broth alone was used as a control for subsequent untargeted metabolomics analysis. This culture was incubated at 37°C for 7 days with continuous agitation at 200 rpm. After incubation, an inoculum comprising 5% (v/v) of the starter culture was transferred to a 2 L fermentation broth of tryptic soy broth for scaled-up fermentation. The fermentation process was maintained at 37°C with shaking at 90 rpm for 20 days. To obtain cell-free crude extracts, cellular debris was removed by centrifugation at 4000 rpm for 10 minutes at 25°C, followed by filtration through a 0.2 µm nylon filter. The resulting cell-free supernatant was subsequently lyophilized 4°C stored at 4°C until solvent extraction. Lyophilized extracts of *B. licheniformis* OM182835 and *M. tuberculosis* H37Rv were reconstituted in methanol. Subsequently, the methanolic crude bacterial extracts were evaporated in a fume hood at ambient temperature and stored at 4 °C for further analysis, as described previously [6]. In brief, crude extracts from *B. licheniformis* OM182835 and *M. tuberculosis* H37Rv were initially prepared by dissolving in 100% dimethyl sulfoxide (DMSO) and subsequently diluted to 10% with distilled water. The extracts

were mixed by vortexing and filter-sterilized through a 0.2-µm nylon filter. For tentative metabolite identification, 2 mg of each extract was dissolved in 1 mL of HPLC-grade methanol.

Untargeted metabolomics

Liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-MS-QTOF) analysis was performed as previously described by Nyambo et al. (2024), with modifications to the chromatographic and mass spectrometric conditions. Briefly, high-resolution mass spectra were acquired using a Waters QTOF mass spectrometer equipped with an electrospray ionization (ESI) source operated in positive ion mode. Chromatographic separation was carried out on a Kinetex C18 column maintained at 60°C. The mobile phase consisted of (A) water containing 0.1% formic acid and (B) methanol containing 0.1% formic acid. The gradient elution program was as follows: 0–0.5 min, 0% B; 0.5–12.0 min, linear increase to 100% B; 12.0–13.0 min, held at 100% B; 13.0–13.1 min, returned to 0% B; and 13.1–15.0 min, re-equilibration at 0% B. The flow rate was set at 0.35 mL/min, and the injection volume was 0.5 µL. Mass spectrometric data were acquired over the m/z 100–1200 range with a scan time of 0.2 s. The instrument was operated in V-mode with a resolving power of approximately 30000–40000 full-width at half maximum (FWHM). Data acquisition was performed using data-independent acquisition with alternating low and elevated collision energies. The low-energy function was acquired at 4–6 eV, while the high-energy function employed a collision energy ramp from 15 to 100 eV to generate fragment ions. A reference mass was continuously infused for real-time mass correction. Source parameters included a source temperature of 120°C and a desolvation temperature of 350°C, with desolvation gas flow set to 600 L/h and cone gas flow maintained at 50 L/h. Nitrogen was used as both desolvation and cone gas. All solvents were sonicated for 10 min prior to analysis.

Feature extraction and annotation

Raw LC-MS/MS data were processed using MZmine (version 4.8.0) [39]. Feature detection was performed using the built-in processing wizard with a mass-to-charge (m/z) detection range of 100–1200 [40]. The MS1 and MS2 mass tolerances were set to 0.01 and 0.025 Da, respectively, and $[M + H]^+$ was selected as the primary adduct. A minimum peak height threshold of 10,000 was applied for feature selection. The resulting feature table and MS/MS spectra were exported for feature-based molecular networking (FBMN) analysis using the GNPS platform [41]. Molecular networks were generated to group structurally related metabolites based on their MS/MS spectral ion tolerance of 0.025 [6]. The putative molecular formulae and structural annotations were predicted using SIRIUS software. Metabolite annotation was further conducted with an m/z error margin of 5 ppm, and the results were compared with m/z values from the NPAtlas and COCONUT databases. To enhance structural interpretation, molecular networks were further explored using GNPS-based enrichment workflows, facilitating the tentative identification of metabolite families and their structurally related analogs.

Determining the lowest working concentration through cytotoxic activity

The minimum effective concentration for the extracts was determined by assessing the viability of Raw264.7 macrophages, as previously described [42]. The macrophages were cultured in DMEM with high glucose, supplemented with 10% fetal bovine serum (FBS) (Gibco), and seeded at a density of 10×10^3 cells per well. Following a 24-hour incubation at 37°C with 5% CO₂, the cells were exposed to various concentrations of metabolites, H₂O₂ (500-7.8125 µM), and LPS (100-3.125 ng/mL), with treatments incubated for an additional 24 hours (Tapfuma et al., 2023). Cell viability was evaluated using the MTT assay. The original medium in each well was replaced with 200 µL of a 0.5 mg/mL MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), followed by a 4-h incubation. Absorbance was subsequently measured at 570 nm using a spectrophotometer (FLUOstar Omega, BMG Labtech, Germany). Cell viability was normalized to that of DMSO (vehicle)-treated cells.

Reactive Oxygen Species (ROS) assay

Intracellular production of reactive oxygen species (ROS) was assessed utilizing a fluorometric ROS kit (Sigma-Aldrich, Catalog Number: MAK143), as previously described [43]. Briefly, RAW264.7 macrophage cells were seeded into 96-well plates at a density of 10×10^3 cells per well and allowed to adhere for 24 h under standard conditions (37°C, 5% CO₂). The metabolite concentration (15 µg/mL) used in this study was determined using the MTT cell viability assay. RAW264.7 macrophage cells were exposed to 15 µg/mL of each metabolite for either one or two hours. For the one-hour exposure, cells received either a single treatment (D1 or BLM) for one hour or two sequential treatments of 30 minutes each (double treatment). For the two-hour exposure, cells underwent either a continuous two-hour treatment or two sequential treatments of 1 h each. Cells treated with 500 µM H₂O₂ served as a positive control. Following treatment, 100 µL of the Master reaction mix was added to the cells, and they were incubated at 37°C with 5% CO₂. The fluorescence intensity of the cells was measured using a fluorescence multi-well plate reader (FLUOstar Omega, BMG Labtech, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Nitric Oxide (NO) production assay

Nitric oxide production was assessed by quantifying intracellular nitrite levels using the Griess reaction (Sigma-Aldrich, Catalog Number: MAK367) according to the manufacturer's protocol. Briefly, Raw264.7 macrophage cells were seeded at a density of 1×10^6 per experimental condition and treated with 15 µg/mL of metabolites from *M. tuberculosis* H37Rv (D1) or *B. licheniformis* OM182835 (BLM). For sequential treatment, cells were first sensitized with D1, then treated with BLM. Lipopolysaccharide (LPS; 100 ng ml⁻¹) was the positive control, while untreated cells served as the baseline control. Post-treatment, cells were detached using 1X trypsin, and the resulting macrophage cell pellet was collected

by centrifugation at 1200 rpm for 5 minutes. The pellet was then resuspended in 100 μ L of ice-cold Nitrite Assay Buffer, incubated on ice for 10 minutes, and centrifuged at $10,000 \times g$ for 5 minutes at 4°C. The supernatant was aliquoted into each well and adjusted to a final volume of 100 μ L with Nitrite Assay Buffer. In this assay, nitrite was first reduced to nitric oxide by sequentially adding Griess Reagent I (10 μ L), Griess Reagent II (10 μ L), and Nitrite Assay Buffer (80 μ L), then mixing and incubating for 10 minutes at room temperature. This nitric oxide subsequently reacted with 10 μ L of Griess Reagent II to form a stable product, quantified by measuring absorbance at 540 nm [44]. Equal volumes of 10 μ L of Griess Reagent I and 90 μ L of Nitrite Assay Buffer were used as the control.

Mitochondrial programming

To investigate the effects of secondary metabolites from *M. tuberculosis* H37Rv (D1) and *B. licheniformis* OM182835 (BLM) on mitochondrial regulation and reprogramming, mitochondrial respiration was evaluated as described previously [45]. Briefly, Raw264.7 macrophages were seeded at a density of 75×10^4 cells per well in a 24-well plate. The experimental treatment conditions included the administration of 15 μ g/mL per 10×10^3 cells of metabolites for two hours; separate exposure to *M. tuberculosis* H37Rv (D1) for two hours; exposure to BLM for two hours; and treatment of *M. tuberculosis* D1-sensitized Raw264.7 macrophages for one hour followed by a one-hour exposure to BLM. Post-treatment, the media were removed, and the cells were detached by adding 1X trypsin to the wells and incubating for 15 minutes. Subsequently, fresh media were added, and the cells were allowed to recover for 30 minutes. The cell pellet was then collected and resuspended in 2 mL of mitochondrial respiration (MIR05) medium. Mitochondrial reprogramming of the treated cells was assessed using high-resolution respirometry on the Oroboros O2K system (Oroboros Instruments, Innsbruck, Austria). Modulation and reprogramming of mitochondrial function resulting from treatment with microbial extracts involved the injection of substrates, uncouplers, and inhibitors [46,47]. Briefly, digitonin (16.0 μ g/mL) was added to permeabilize the cell membranes, followed by the introduction of substrates for complex I leak, including pyruvate (5 mM) and malate (2 mM). Stimulation of ATP synthase and Complex I-linked OXPHOS was achieved by adding ADP (1 mM). The integrity of the outer mitochondrial membrane was evaluated by injecting cytochrome C (10 μ M). The capacity of the electron transfer system (ETS) was determined by gradually titrating carbonyl cyanide m-chlorophenylhydrazone (0.5 μ M). To evaluate uncoupled Complex I-linked OXPHOS, glutamate (10 mM) was introduced, while succinate (10 mM) was added to assess uncoupled complex I and complex II. Subsequently, respiration via the F, N, and S pathways was stimulated by adding octanoylcarnitine (0.5 μ M). The electron flow through the ETS was inhibited by the addition of rotenone (1 μ M), a complex I inhibitor. Following this, antimycin A (2.5 μ M) was added to inhibit complex III, thereby allowing determination of residual oxygen consumption (Rox). Finally, the activity of complex IV was evaluated by adding ascorbate (2 mM) along with tetramethylphenylenediamine (TMPD) (0.5 mM), followed by sodium azide (200 mM).

RNA Extraction and Bulk RNA Sequencing: Data Processing and Differentially Expressed Genes (DEGs)

RAW264.7 cells were cultured in T25 flasks at a density of 25×10^4 cells per flask and incubated for 24 hours. Subsequently, the cells were exposed to 15 $\mu\text{g/mL}$ of bacterial metabolites per 10^3 RAW264.7 cells for 2 hours. RNA extraction from the treated cells was conducted using the Direct-zol RNA Kit Miniprep (ZYMO RESEARCH, #R2053-A). The purity of the extracted RNA was assessed using a Thermo Scientific Nanodrop One (Wilmington, Delaware, USA). Library preparation, including rRNA depletion, and sequencing were performed at CD Genomics on an Illumina PE150 platform, yielding a total of 60 million reads per sample. RNA-seq data preprocessing was executed on the CHPC Lengau cluster, as previously described (Piper et al., 2022). Transcript abundances were quantified using Salmon v. 1.9.0 against the *Mus musculus* (GRCm39, release 115) [49]. The EdgeR v4.6.3 package in RStudio v 2026.01.0 + 392 was used to identify differentially expressed genes (DEGs) using pairwise comparisons (D1 vs. control; D1BLM vs. control). The trimmed mean of M-values (TMM) method was applied for library normalization. DEGs were selected based on a False Discovery Rate (FDR) ≤ 0.01 and a $\log_2$ fold change ≥ 1 for the comparisons. Principal component analysis was conducted on the top 500 genes to elucidate the differences between samples. Functional enrichment analysis was performed using clusterProfiler v4.16.0 [50]. Insights into molecular interactions were further enhanced through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Gene Ontology (GO) analysis was conducted to identify significantly enriched ontologies, encompassing biological processes, molecular functions, and cellular components. Concurrently, KEGG analysis was employed to identify significantly enriched signaling and metabolic pathways [51,52]. The significance thresholds for this analysis were set at FDR ≤ 0.05 and $\log_2$ fold change ≥ 1 .

Statistical analysis

All experiments were conducted in biological and technical triplicates, with results expressed as mean $\pm$ standard deviation. Statistical analyses were performed using GraphPad Prism 8 (v8.0.1; GraphPad Software, La Jolla, CA, USA). Statistical significance for ROS and NO production was assessed using two-way ANOVA followed by Tukey's multiple-comparisons correction. To examine the modulation of mitochondrial respiration, a two-way ANOVA was employed, with treatment condition and respiratory state as factors. Pairwise comparisons between treatment groups within each respiratory state were performed using Tukey's multiple comparisons test. Significance is indicated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

Soil characterization in this region indicated a highly acidic profile, with a pH of 3.56. The electrical conductivity (EC) values were low (1 dS/m), suggesting minimal levels of dissolved salts and ions. Nutrient content was significantly low, as evidenced by total carbon (TC) at 0.35 g/kg, total nitrogen (TN) at 0.007 g/kg, and permanganate oxidizable carbon (POXC) at 95 mg/kg. The elevated carbon-to-nitrogen (C:N) ratio of 41 further highlights the deficiency of soil organic matter, which correlates with low nutrient availability and poor fertility.

Taxonomic classification of mine tailings and MAGs

Gold mine tailings were characterized by highly acidic, nutrient-deficient soil conditions, which may be associated with low microbial diversity (Fig. 2A). The microbial community was predominantly composed of members of the genus *Bacillus*, with *Bacillus coagulans* (*B. coagulans*) being the most prevalent species (Fig. 2A). The identified taxa have been previously documented to withstand high metal concentrations and acidic environments. In alignment with the extreme environmental conditions present in the mine tailings (Baker et al., 2006)(Baker et al., 2006), only a limited number of MAGs were recovered. Of the three MAGs reconstructed from the gold mine tailings, two exhibited high quality ($\geq 90\%$ completeness and $\leq 5\%$ contamination), while one was classified as medium quality, with ($< 90\%$ completeness and 8.9% contamination), according to the cutoff criteria established by Bowers et al. (2017) (Bowers et al., 2017). Taxonomic classification using GTDB-Tk assigned these MAGs to the genera *Bacillus* and *Clostridium* (Fig. 2B), specifically *Bacillus amyloliquefaciens* SAMN61110746 (*B. amyloliquefaciens*) (bin.1), *B. coagulans* SAMN61110747 (bin.2), and *Clostridium perfringens* SAMN61110745 (*C. perfringens*) (MAG bin.3). The genome size of the MAGs ranged from 3.2 Mbp to 4.9 Mbp (Table S1). Cultivation-based enrichment of bacteria from the gold mine tailings yielded a *Bacillus*-dominated co-culture, from which two high-quality MAG bins were reconstructed ($\geq 90\%$ completeness and $\leq 5\%$ contamination). These genomes were taxonomically assigned to *Bacillus simplex* (*B. simplex*) SAMN36381075 MAG (bin.001) and *B. licheniformis* SAMN36381076 MAG (bin.002), with genome sizes of 5.9 Mbp and 4.2 Mbp, respectively. Further characteristics of all the MAGs are summarized in Table S1.

A.

B.

Functional annotation of assembled MAGs

The functional analysis of MAGs derived from tailings indicates that *B. amyloliquefaciens* (bin.1) contributes to nitrogen fixation, carbohydrate metabolism, and primary carbon turnover within the soil (Fig. 3). The CAZy annotation reveals that bin.1 encodes essential enzymes involved in the degradation of complex cellulose and starch. Moreover, bin.1 contains genes associated with chemolithotrophic metabolism, as evidenced by the presence of genes involved in nitrogen cycling, including denitrification and ammonia oxidation. Furthermore, the presence of genes encoding reductase enzymes suggests their role in adapting to the high metal tailings niche, characterized by fluctuating redox conditions (Figures S2). Similarly, *B. coagulans* (bin.2) encodes genes associated with denitrification, nitrification, and nitrate reduction (Fig. 3). In contrast, *B. coagulans* (bin.2) encodes genes responsible for methanogenesis. The co-occurrence of these pathways suggests that bin.2 may participate in methane cycling, likely at redox transition zones where both electron donors and acceptors are variably available (Figures S2). In contrast to the other bins, bin.3 exhibits anaerobic carbon turnover, a metabolic process associated with the conversion of acetyl-CoA and pyruvate, resulting in ethanol production. The

fermentative energy present in bin.3 may facilitate the production of acetate and hydrogen, which are utilized by bacteria that adopt a methanogenic lifestyle. The *B. licheniformis* SAMN36381076 MAG (bin002) reveals that the bacterium encodes genes responsible for the catabolism of carbohydrate complexes (Fig. 3). Additionally, *B. licheniformis* SAMN36381076 is observed to encode genes involved in oxyanion cycling, including nitrate (Fig. 3). The bacterium encodes a gene responsible for arsenite oxidation, as well as peroxidase and catalase enzymes. These genetic features are consistent with adaptation to heavy-metal mine-tailings environments (Fig. 3, Figure S2, and Figure S3).

This study evaluated the functional genomics of the enriched *B. licheniformis* SAMN36381076, focusing on the production of bioactive compounds that are potentially significant in mine tailings. A total of 13 biosynthetic gene clusters (BGCs) were tentatively identified and associated with the production of secondary metabolites (Figure S1). The genome revealed a diverse array of BGCs, including those for β -lactones, terpenes, thiopeptides, lasso peptides, ribosomally synthesized and post-translationally modified peptide (RiPP) recognition elements (RRE), Type III polyketide synthases (T3PKS), class II lanthipeptides, NI-siderophores, tRNA-dependent cyclodipeptide synthases (CDPS), and non-ribosomal peptides (NRPS). The identified siderophores, exhibiting 60% homology, are known to facilitate bacterial acquisition of metal ions, which are subsequently incorporated into intracellular respiratory pathways (Fig. 4). The BGC analysis tentatively identified gene clusters responsible for the biosynthesis of lichenicidin VK21 A1 and A2, as well as thermoactinoamide A, with 100% homology. Clusters 1.1, 5.1, 13.1, 15.1, 23.1, and 36.1 demonstrated varying degrees of homology, showing sequence similarities of 53%, 7%, 50%, 60%, and 66% to the following metabolites: fengycin, butirosin A and B, schizokinen, pulcherriminic acid, and bacillibactin (comprising bacillibactin E and F), respectively. In this context, bacteria that produce lethal antimicrobial agents have developed self-protective mechanisms to avoid self-destruction. Tentative identification of these mechanisms of action indicates resistance to various antimicrobial classes, including macrolides, lincosamides, aminoglycosides, peptide antibiotics, multiple antibiotic resistance, tetracyclines, and phenicol antibiotics (Table S2).

Metabolomic analysis of extracts from *B. licheniformis* OM182835 (BLM) and *M. tuberculosis* H37Rv (D1)

The metabolites tentatively identified from BLM, as depicted in Fig. 5 and Figure S2, encompass a wide array of protonated compound classes, with m/z values ranging from 333.2995 to 1050.9621. Notably, these metabolites include a bacillibactin homolog (m/z 881.6186), surfactin daughter ions (m/z 683.4328, 699.3791, 693.4646), a fengycin A homolog (m/z 730.5416), and a lichenysin A homolog (m/z 1050.9621). Conversely, while the biochemical diversity of the *M. tuberculosis* metabolome is extensive, its structural and functional characterization remains largely unexplored. The tentatively identified metabolites from *M. tuberculosis* H37Rv (D1) within the molecular network include glycolipid homologs of Phthiocerol Dimycocerosate (PDIM) A at m/z 1110.7912. This homolog is associated with another PDIM homolog (m/z 1042.8030) and daughter intermediates (m/z 974.8150). The MS/MS fragment spectrum of these tentatively identified homologs reveals a predominant phthiocerol core fragment (m/z

226.95) (Figure S11, Figure S12, and Figure S13) [54] and characteristic polymethyl-branched mycoserosic acid tail fragments (m/z 430.91 and 362.92) [55].

Surfactin

Fengycin

Bacillibactin

Investigation into the modulation of ROS, NO, and the perturbation of mitochondrial function

This study investigated the early modulation of ROS and NO production in Raw264.7 macrophages following exposure to metabolites derived from *M. tuberculosis* H37Rv (D1) and *B. licheniformis* OM182835 (BLM) (Figs. 6A, Fig. 6B, Figure S5, and Figure S6). The treatment concentration was 15 $\mu\text{g/mL}$ of extracts, which maintained 100% cell viability in Raw264.7 macrophages, with viability $\geq 80\%$ (Figure S7). The results indicate a time-dependent increase in the ROS response of Raw264.7 macrophages to these treatments. After 1 hour, there was no significant increase in ROS production compared to untreated cells, including ($p = 0.9232$), BLM ($p = 0.5723$), and D1 + BLM ($p = 0.8820$) (Fig. 6A). Treatment with D1 did not significantly enhance ROS production compared to untreated cells ($p = 0.5723$; Fig. 6A). Conversely, treatment with BLM resulted in a significant increase in ROS production relative to untreated cells ($p = 0.0022$). A similar increase in ROS production was observed following pretreatment with D1 and subsequent exposure to BLM (D1 + BLM) compared to untreated cells ($p = 0.0044$). Additionally, direct comparisons between treatment groups revealed that metabolites in BLM induced a significantly greater increase in ROS production after 2 hours than D1 alone ($p = 0.0439$). However, the D1 + BLM treatment (pretreatment with D1 followed by BLM exposure) did not differ from D1 treatment alone in ROS production ($p = 0.0845$).

To further elucidate the differential regulation of early cellular responses by metabolites in D1 and BLM, NO production was assessed at 1 and 2 hours following stimulation with D1, BLM, D1 + BLM, or LPS. Treatment with either D1 or BLM alone did not significantly alter NO production compared with untreated Raw264.7 macrophages (Fig. 6B). Conversely, the combined treatment, involving pretreatment with D1 followed by exposure to BLM (D1 + BLM), resulted in a significant increase in NO production after 1 hour, followed by a reduction after 2 hours. These findings indicate that co-stimulation of Raw264.7 macrophages with D1 + BLM induces a rapid, transient response.

To examine the modulation of mitochondrial respiratory responses, RAW264.7 macrophages were treated with metabolites derived from *M. tuberculosis* H37Rv (D1), *B. licheniformis* OM182835 (BLM), or pretreated with D1 followed by exposure to BLM (D1 + BLM). Treatment with D1 resulted in mitochondrial dysregulation, as indicated by significantly reduced routine respiration compared with untreated cells (Fig. 6C). To further characterize the metabolic respiratory impairment, substrate-linked respiratory states were evaluated. D1 treatment significantly decreased malate dehydrogenase-linked respiration

relative to that with BLM and also significantly reduced pyruvate dehydrogenase-linked respiration relative to that in untreated cells (Fig. 6C). In contrast, D1 + BLM treatment did not significantly affect routine respiration, malate dehydrogenase-linked respiration, or pyruvate dehydrogenase-linked respiration compared to that in untreated cells. Direct comparisons between D1 and D1 + BLM revealed no statistically significant difference in routine respiration, malate dehydrogenase-linked respiration, or pyruvate dehydrogenase-linked respiration, suggesting partial modulation of RAW264.7 macrophages rather than complete restoration. Notably, D1 + BLM treatment significantly enhanced Complex I-linked OXPHOS compared to untreated cells, indicating that co-exposure to BLM redirects or enhances Complex I-dependent oxidative phosphorylation. Additionally, the findings suggest modifications in succinate-linked respiration, as evidenced by significantly higher Complex II-linked OXPHOS in D1 + BLM-treated cells compared with BLM-treated cells. However, the comparison between D1 + BLM and untreated cells showed no significant difference, indicating selective mitochondrial remodeling.

B.

A.

C.

Transcriptional modulation of Raw264.7 macrophage cells

RNA sequencing was performed to elucidate the molecular reprogramming underlying these phenotypic alterations. Principal component analysis demonstrated that distinct transcriptional responses in the samples were uniquely associated with the treatments (BLM, D1, D1 followed by BLM exposure (D1BLM), and untreated control) (Figure S8A). RAW264.7 macrophage cells treated with *M. tuberculosis* H37Rv (D1) exhibited upregulation of genes, including *Acod1* (proinflammatory), *Rpl28-ps1*, *Tpt1-ps3*, *Gm7367*, *Mrip-ps*, and *Erhrt-ps*. Conversely, a key regulator of β -oxidation (*Cpt1b*), along with *Pakap*, *Atp6-ps*, *Eif4a-ps4*, and *Gm49359*, was downregulated following D1 treatment (Fig. 7A). Functional enrichment analysis indicated the enrichment of genes associated with palmitoyltransferase and O-acyltransferase activities, both of which are regulated by *Cpt1b* (Figure S8C). Additionally, KEGG pathway enrichment analysis identified *Cpt1b* as a central node, linking fatty acid metabolism to AMPK signaling, PPAR, and glucagon signaling pathways (Figure S8B). Furthermore, the activity of proton-transporting ATP synthase was enriched and associated with *Atp6-ps*. In contrast, the proinflammatory gene *Acod1* was associated with enrichment for carboxy-lyase activity, suggesting activation of immunometabolic pathways associated with itaconate production (Figure S8C). These RNA sequencing results corroborate and expand on insights into dysregulated mitochondrial metabolism (Fig. 6E).

A.

B.

A.

B.- .

In contrast, RAW264.7 macrophage cells stimulated with metabolites derived from *M. tuberculosis* H37Rv (D1), followed by treatment with metabolites from *B. licheniformis* OM182835 (BLM), exhibited upregulation of several genes, including *Scd1*, *Bnip3*, *Epm2a*, *Atp6v0d2*, *Ptpn14*, *Mturn*, *Efcab6*, *Tmem265*, *Rufy4*, *Zfp708*, and *Pira2*. Conversely, genes such as *Pakap*, *Gcat*, *Rpl21-ps15*, *Atp6-ps*, *Semp2l2a*, *Gm28439*, and *Gm8495* were downregulated (Fig. 7B). Functional enrichment analysis indicated enrichment of genes associated with autophagy-related and redox pathways (Fig. 7C). Transcripts associated with responses to oxygen deprivation, including *Bnip3* and *Map1lc3a*, were enriched. Simultaneously, upregulation of pathways involved in the positive regulation of macroautophagy (*Rufy4*, *Epm2a*, and *Bnip3*) and in autophagosome assembly (*Epm2a*, *Map1lc3a*) were enriched, suggesting a shift towards autophagic processes. Additionally, the enrichment of microtubule-associated processes (*Ndr1*, *Spaca9*, and *Efcab6*) along with structural components of the axonemal microtubule doublet inner sheath (*Efcab6*) suggests cytoskeletal remodeling. Furthermore, brown fat cell differentiation was enriched (*Selenbp1* and *Scd1*), further indicating reprogramming towards lipid remodeling and the increase in ROS observed from treatment in Fig. 6B and Fig. 6E. Notably, the proton-transporting V-type ATPase complex was enriched (*Atp6v0d2* and *Atp6-ps*), suggesting modulation in vesicular acidification, which is critical for the maturation of the autophagosome.

C.

Discussion

Bioactive natural products extracted from extremophilic microbes have gained increasing attention as potential sources of antimicrobial agents [56,57]. Numerous organic compounds derived from these sources have demonstrated inhibitory effects against various drug-resistant pathogens, indicating their potential as foundational elements for the development of novel therapeutics [6,58]. The findings of this study corroborate previous research, which has shown that gold mine tailings are characterized by high acidity, limited nutrient content, and elevated concentrations of heavy metals such as cadmium (Cd), lead (Pb), iron (Fe), nickel (Ni), and zinc (Zn) [59–61]. This observation aligns with the characterization of *B. licheniformis*, *B. amyloliquefaciens*, and *B. coagulans*, MAGs identified in the gold mine tailings. These species exhibit tolerance to high concentrations of heavy metals and acidic pH conditions, as evidenced by their genomes, which encode various peroxidases, catalases, and siderophores. These genomic features may support bacterial metal chelation, potentially facilitating survival in environments where the oxidation states of heavy metals in mine tailings are variable [62–66]. At the genomic level, *B. licheniformis* SAMN36381076, *B. amyloliquefaciens*, and *B. coagulans* encode genes related to nitrogen cycling and carbon turnover. These findings are consistent with previous studies that utilized *B. licheniformis* and *B. amyloliquefaciens* to enhance nitrogen availability and carbon turnover in soil [63,67,68].

Understanding microbial metabolite resistance mechanisms is essential for identifying potential bioactive products, determining their modes of action, and complementing untargeted metabolomics [69–71]. The tentative genomic and metabolomic analysis suggests that *B. licheniformis* may produce fengycin, lichenysin, lichenicidin, and bacillibactin [72,73]. Notably, the homology among these compounds is less than 70 %, suggesting that *B. licheniformis* SAMN36381076 may produce novel derivatives within these lipopeptide classes. The antimycobacterial efficacy of these metabolites has been previously reported against *M. smegmatis* [71]. Additionally, this study supports recent findings that *Bacillus* spp. secretes siderophores, such as bacillibactin and schizokinen [74–76].

Building on the previously reported antimycobacterial activity of secondary metabolites derived from *B. licheniformis* OM182835 against *M. tuberculosis* H37Rv (MIC = 1.25 mg/mL) and *M. smegmatis* mc²155 (MIC = 0.3125 mg/mL) [6], this study investigates the potential modulation of macrophage bioenergetic responses by these metabolites when induced by *M. tuberculosis* H37Rv-derived metabolites. The existing literature highlights the pivotal role of macrophage mitochondrial metabolism in host defense, including ROS production, inflammatory signaling, and cell death fate during *M. tuberculosis* infection [77–79]. In this study, exposure of Raw264.7 macrophages to metabolites from *M. tuberculosis* H37Rv (D1) did not significantly increase ROS production, consistent with previous studies that *M. tuberculosis* virulence factors dampen macrophages ROS responses, part of *M. tuberculosis* immune evasion [80,81]. In contrast, *B. licheniformis* OM182835 (BLM) treatment significantly increased ROS production after 2 hours, and a significant increase was also observed in the D1 + BLM treatment. These findings suggest early stimulation of ROS responses by metabolites from BLM or following D1 exposure. The biphasic NO response observed after the pretreatment with D1 and subsequent exposure to BLM (D1 + BLM) is consistent with studies that have suggested an association of *B. licheniformis* beneficial bisphasic immunometabolic regulation.

In this study, post-D1 treatment showed a reduced routine respiration, suggesting a potential negative regulation of basal mitochondrial oxygen consumption. Furthermore, a significant reduction in malate dehydrogenase-linked respiration was observed compared to BLM and pyruvate dehydrogenase-linked respiration in untreated cells. Since these two pathways (malate dehydrogenase and pyruvate dehydrogenase) generate NADH and allow electron entry into Complex I, these findings suggest that D1 may disrupt the anaplerotic carbon entry into the citric acid cycle [82]. This phenotype was associated with increased expression of the *Acod1* transcript, encoding the rate-limiting enzyme Acod1 (Irg1), a crucial regulator of itaconate production [14]. Since itaconate has been implicated in the regulation of mitochondrial metabolic signaling [83], *Acod1* upregulation may contribute to the suppressed oxidative phenotype observed after D1 exposure. Concurrently, D1 downregulated the *Cpt1b* transcript, which encodes carnitine palmitoyltransferase 1, a crucial regulator of mitochondrial fatty acid β -oxidation [84]. Functional enrichment analysis linked *Cpt1b* to palmitoyltransferase activity, O-acyltransferase activity, fatty acid metabolism, as well as AMP-activated protein kinase (AMPK) signaling glucagon signaling pathways. This transcriptional observation may be associated with diminished oxidative metabolic flexibility and disrupted substrate utilization. Together with the observed decrease in routine and substrate-linked respiration, these findings suggest that D1 induces a shift in macrophages towards a

state of mitochondrial metabolic constraint, characterized by reduced basal respiration, impaired TCA cycle input into OXPHOS, *Acod1* upregulation, and *Cpt1b* downregulation.

Previous research has demonstrated that metabolites from *B. licheniformis* can reprogram host cells infected with *C. perfringens* and positively modulate cell death pathways [85–87]. In this study, macrophages sensitized with D1 followed by BLM (D1 + BLM) displayed a distinct transcriptional and functional phenotype. Notably, D1 + BLM treatment did not significantly alter routine respiration, malate dehydrogenase-linked respiration, or pyruvate dehydrogenase-linked respiration compared with untreated macrophages. However, a comparison between the treatments D1 and D1 + BLM on these respiration parameters was not significant, which may imply a partial reprogramming of mitochondrial respiration.

Moreover, the D1 + BLM treatment significantly enhanced Complex I-linked OXPHOS compared to untreated cells, suggesting that BLM treatment augments Complex I-linked OXPHOS following D1 exposure. This treatment may have also selectively altered succinate-linked respiration, as evidenced by the significantly increased Complex II-linked OXPHOS in comparison to BLM-treated macrophages. These findings are further corroborated by transcriptional profiling of D1 + BLM-treated macrophages, which demonstrated the upregulation of genes associated with autophagy, mitophagy, redox adaptation, vesicular acidification, and lipid remodeling. The BLM treatment following D1 sensitization suggests a stress-adaptive autophagy-linked response, associated with the enrichment of oxygen deprivation response genes such as *Bnip3*, along with macroautophagy and autophagosome assembly pathways involving *Rufy4*, *Epm2a*, and *Map1lc3a*. Additionally, the enrichment of the V-type ATPase complex in D1 + BLM treated macrophages further indicates modulation of vesicular acidification. Collectively, these results suggest a mechanistic association of BLM treatment with autophagy-associated transcriptional remodeling and enhanced cellular stress management. These mechanisms are crucial in *M. tuberculosis* pathogenesis, as the secondary metabolites from *M. tuberculosis* are implicated in disrupting the host's protective mechanisms and facilitating intracellular persistence [88].

Limitations

A comprehensive understanding of the modulation of immune responses may necessitate the infection of macrophages with *M. tuberculosis* H37Rv, followed by treatment with metabolites derived from *B. licheniformis*. This approach aims to ascertain whether these metabolites can mitigate the immunoparalysis induced by *M. tuberculosis*. Furthermore, the compounds present in the extracts were preliminarily identified, and subsequent purification and identification using NMR will yield additional insights into their mechanisms of action. Cytokine profiling may augment the study by providing a comprehensive mechanistic perspective on the restoration of mitochondrial function and the associated cell death pathways.

Conclusion

The tailings generated by gold mining create an extreme environment conducive to the proliferation of bacteria with distinctive metabolic capabilities. These findings suggest that bioactive metabolites from *B. licheniformis* OM182835 modulate macrophage bioenergetic and redox responses following exposure to *M. tuberculosis*-derived metabolites. Future studies using purified compounds, live infection models, cytokine profiling, and pathogen-survival assays are required to define their host-directed therapeutic potential.

Declarations

Ethical Approval:

Ethical approval for this study was granted by the Research Ethics Committee: Biological and Environmental Safety (REC: BES) of Stellenbosch University with reference number BEE-2022-3188. This research was conducted as non-commercial, fundamental research, concentrating on bacteria isolated from gold mine tailings, which are substrates created by human activity. As the study did not involve bioprospecting for commercial or industrial objectives, obtaining a bioprospecting permit under the National Environmental Management: Biodiversity Act was unnecessary. Authorization for access to the sampling site was granted by Nick Botha. The sampling process entailed the manual collection of solid tailings and did not constitute water use under the National Water Act, nor did it involve any listed activity requiring environmental authorization under the National Environmental Management Act (NEMA). The National Environmental Management Act 107 of 1998 (NEMA) functions as South Africa's primary framework legislation for environmental management. It establishes principles for environmental governance and provides the institutional framework for environmental protection, including mechanisms for compliance and enforcement. Within this framework, NEMA mandates Environmental Authorization for certain listed activities. As previously noted, routine scientific sampling of tailings typically does not require such authorization.

Software and code used

<https://github.com/WrightonLabCSU/dram-viz>

<https://github.com/Arkadiy-Garber/MagicLamp>

<https://www.kbase.us/>

Funding:

This work was supported by the South African Medical Research Association Council (SAMRC).

Author Contribution

Conceptualization, V.M., and K.N.; methodology, K.N., V.M., R.R.D.F and GM.; formal analysis, K.N., R.R.D.F.; investigation, K.N., R.R.D.F.; resources, V.M., K.N.; L.S., GM., A.G.L and I.S.N.; writing original draft preparation, K.N.; writing review and editing, V.M., I.S.N., R.R.D.F., B.M., A.G.L., L.B., L.S.; GM., E.G., A.K., M.K., supervision, V.M., A.K., M.K., and L.S.; project administration, K.N.; funding acquisition, V.M., K.N.; L.S and A.G.L. All authors have read and agreed to the published version of the manuscript.

Acknowledgement

The authors express their gratitude to Brendon Mann and Felicia Wells for their invaluable assistance in the library preparation of metagenomics samples. The authors express their sincere gratitude to the Acting Manager of the Property Management, Real Estate Department, City of Ekurhuleni, for their support and assistance.

Data Availability

The datasets generated and/or analyzed in this study are accessible in the Gene Expression Omnibus (GEO) database at [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE348591>] under the accession number GSE348591. This dataset comprises samples treated with BLM (*Bacillus licheniformis*) metabolites (GSM10075683), samples treated with D1 (*Mycobacterium tuberculosis* H37Rv) metabolites (GSM10075684), samples subjected to sequential treatment with D1 metabolites followed by BLM metabolites (GSM10075685), and untreated control samples (GSM10075686).

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Figures

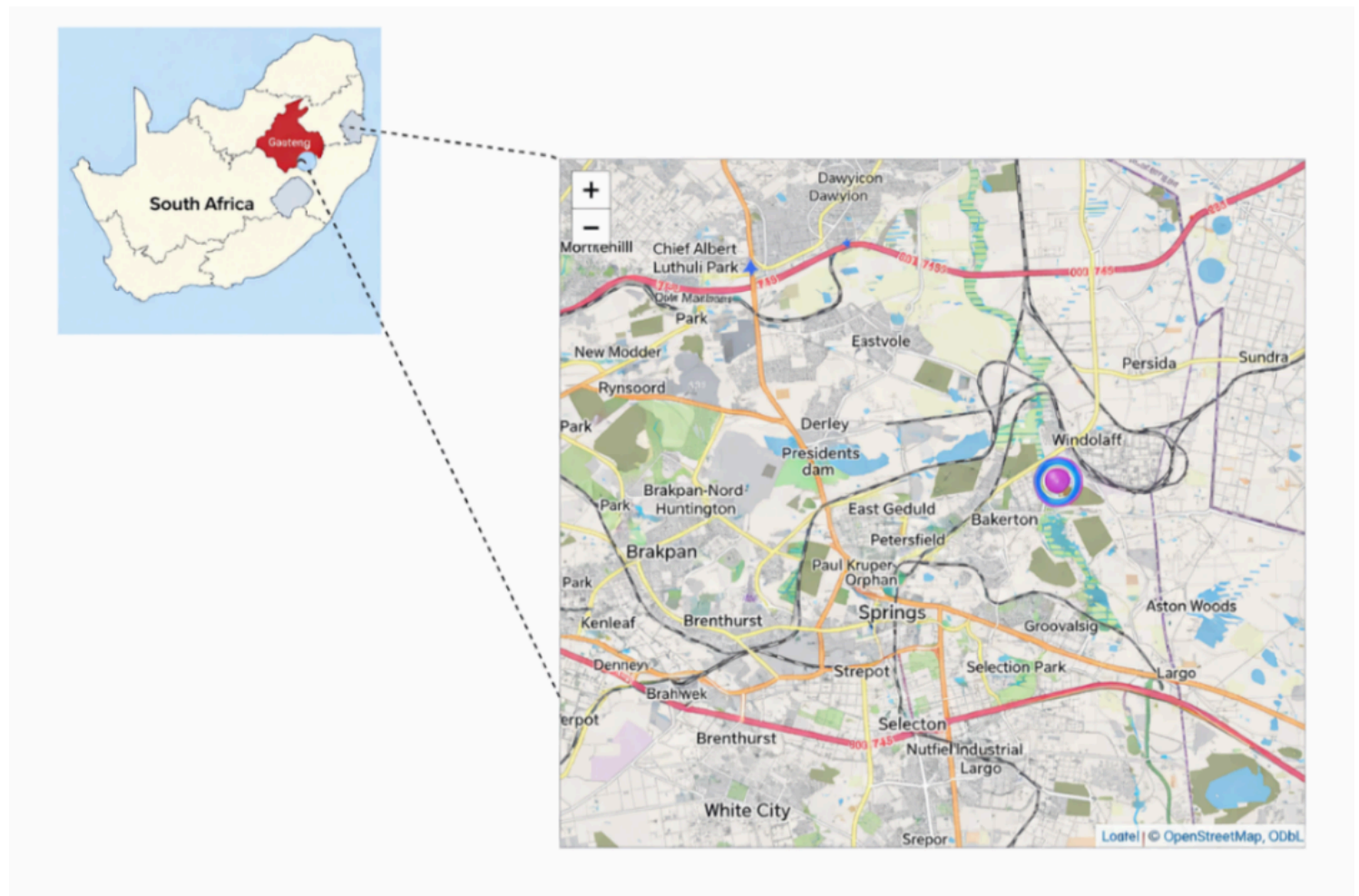


Figure 1

Geospatial representation of the gold mine tailings sampling location.

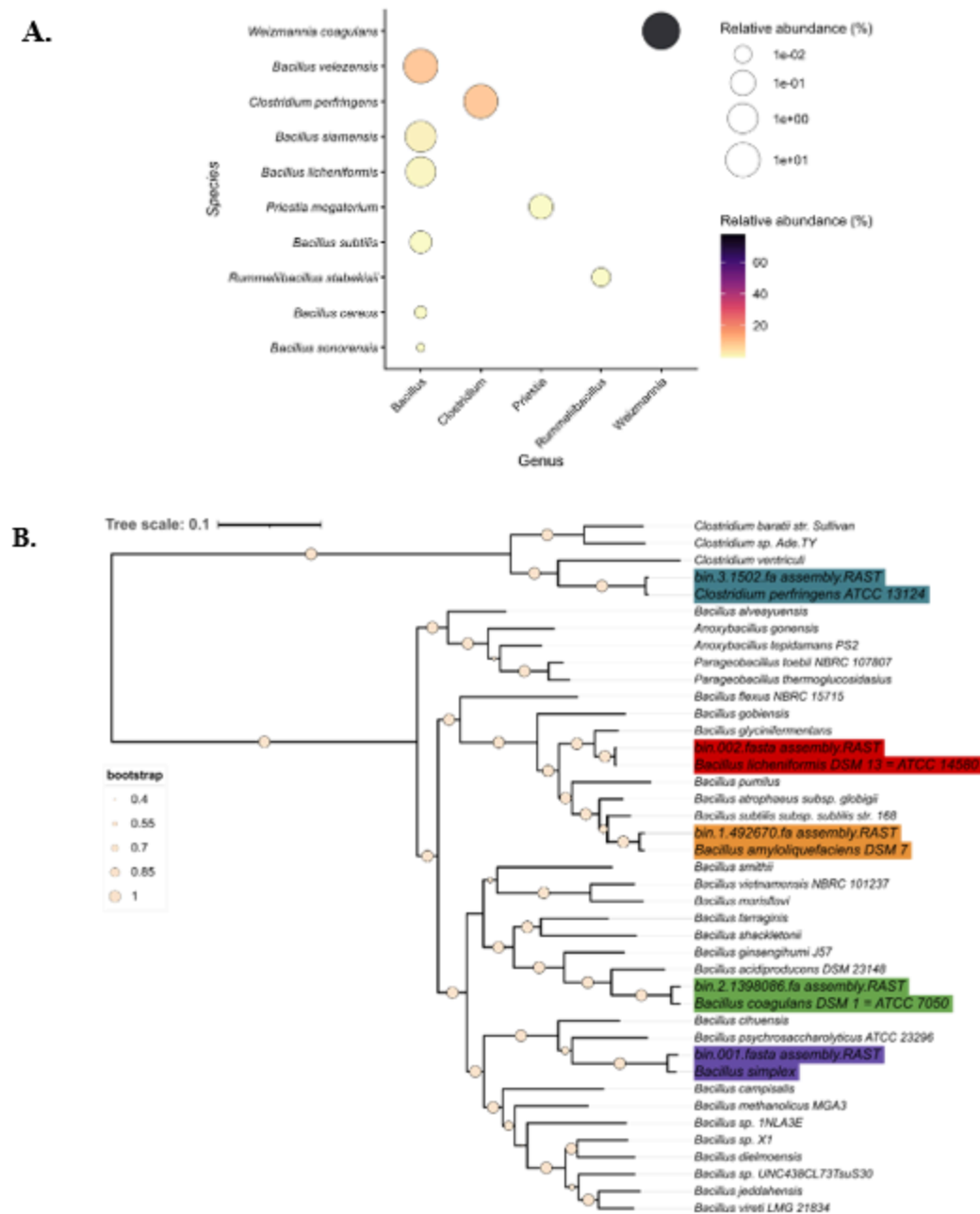


Figure 2

Taxonomic composition and phylogenetic positioning of bacterial MAGs derived from gold mine tailings. **A.** Relative abundance of bacterial taxa within the soil of gold mine tailings. **B.** Phylogenetic placement of the bacterial MAGs reconstructed from the tailings soil sample, alongside their closest relatives. Phylogenetic trees were constructed using the maximum-likelihood method, with bootstrap support values obtained from nonparametric bootstrapping across 100 replicates.

Putative functions

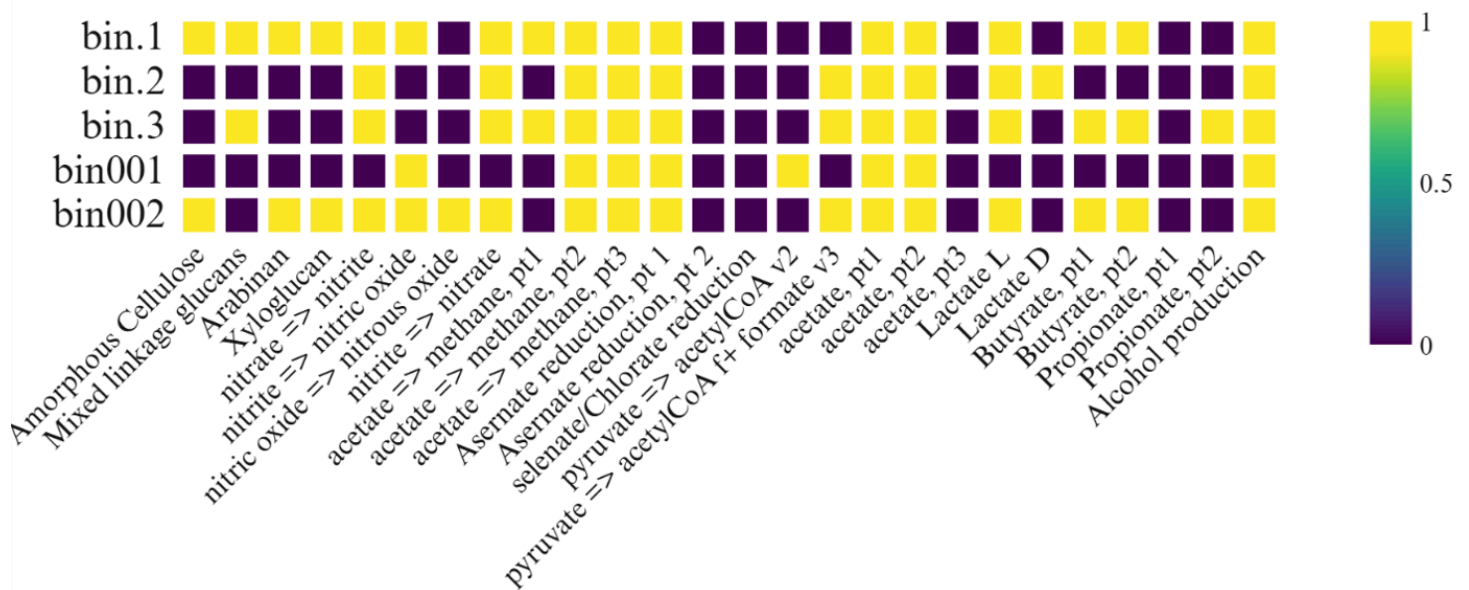


Figure 3

Putative functional capabilities of MAGs and enriched culturable bacteria from tailings soil.

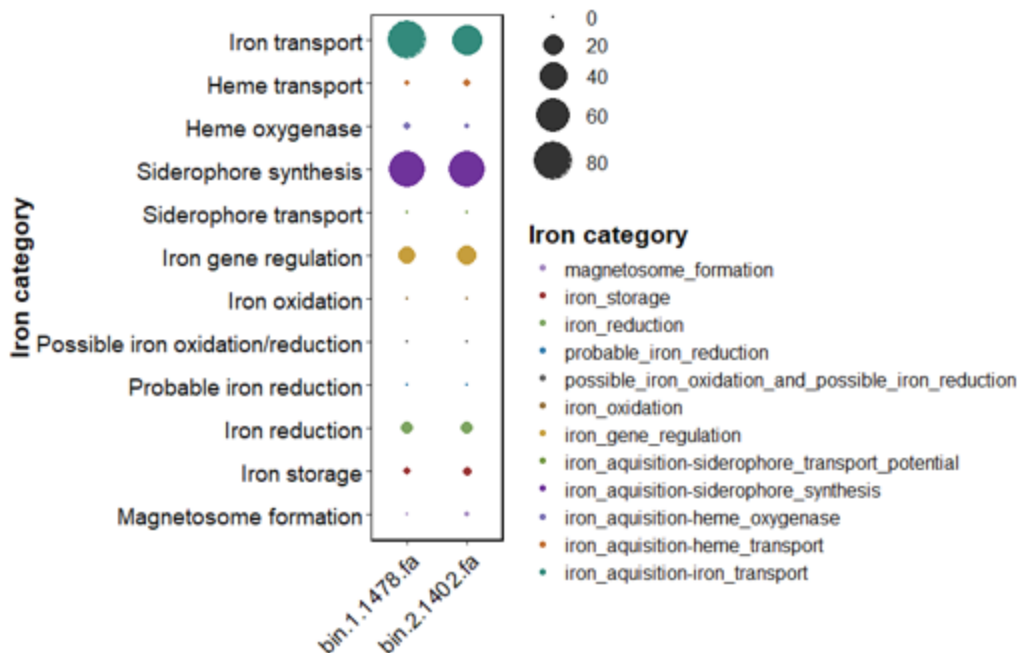


Figure 4

Putative functional culturable bacteria from a heavy metal-rich gold mine tailings niche. Annotated metal acquisition from the genomes of bacteria (*B. licheniformis* SAMN36381076 (bin.2.1478) and *B. simplex* SAMN36381075 (bin.1.1402)).

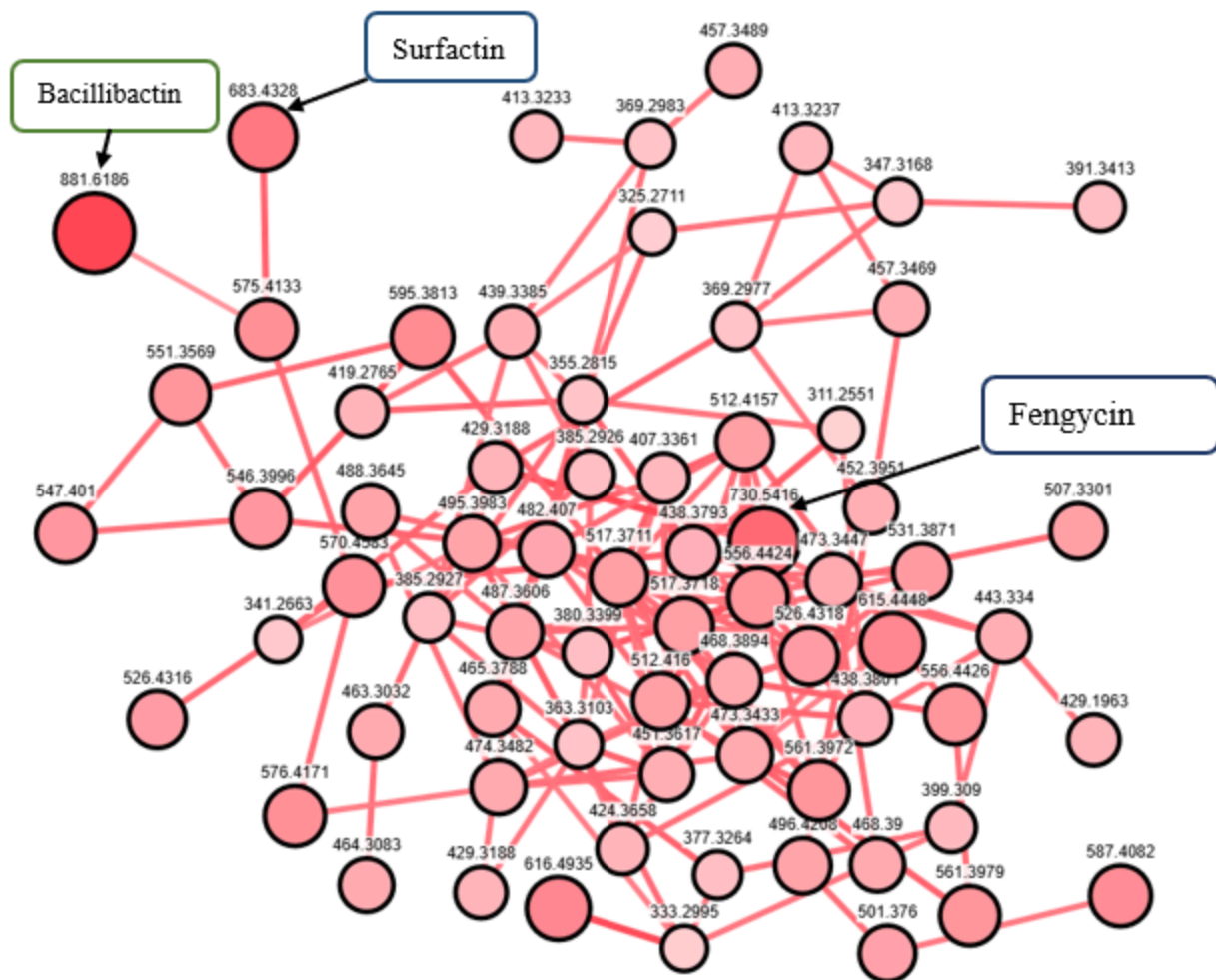


Figure 5

Tentatively identified metabolites derived from *A. B. licheniformis* OM182835 (BLM).

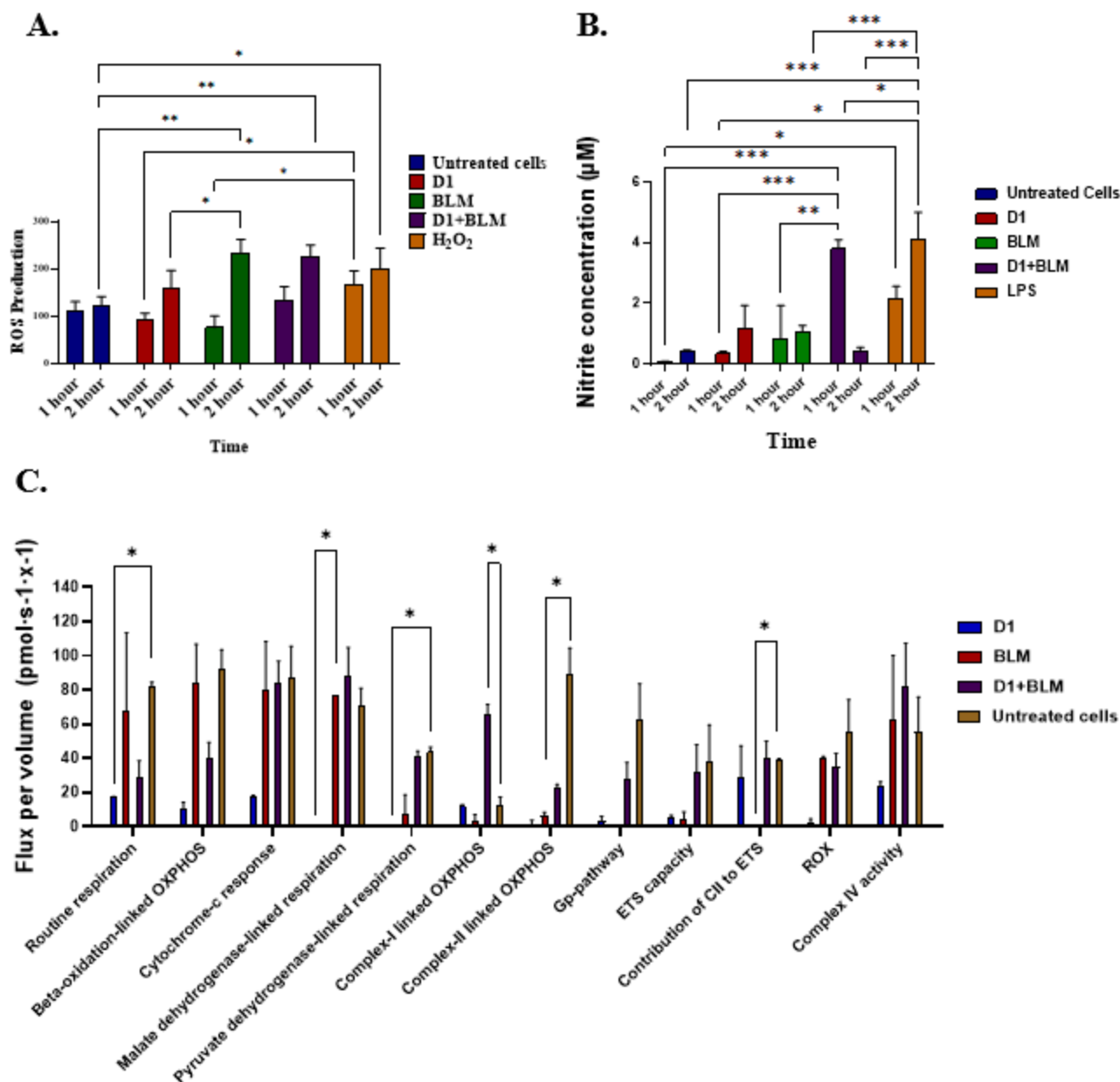


Figure 6

Modulation of ROS, NO, and mitochondrial respiratory responses in RAW 264.7 macrophages by bacterial secondary metabolites. RAW 264.7 macrophages were stimulated with secondary metabolites derived from *M. tuberculosis* H37Rv (D1) and *B. licheniformis* OM182835 (BLM). **A.** Reactive oxygen species (ROS) production was measured 1-2 h after stimulation. **B.** Nitric oxide (NO) production was measured 1-2 h after stimulation. **C.** RAW 264.7 macrophages were treated with D1, BLM, or D1 followed by BLM exposure (D1+BLM), and cellular respiration was assessed across defined respiratory states. Respiratory activity is expressed as flux per volume. All experiments were conducted in triplicate, and the results are presented as mean $\pm$ standard deviation. Statistical significance for ROS and NO production was assessed using two-way ANOVA followed by Tukey's multiple-comparisons correction. To examine the modulation of mitochondrial respiration, a two-way ANOVA was employed, with treatment condition and respiratory state as factors. Pairwise comparisons between treatment groups within each

respiratory state were performed using Tukey's multiple comparisons test. Significance is indicated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

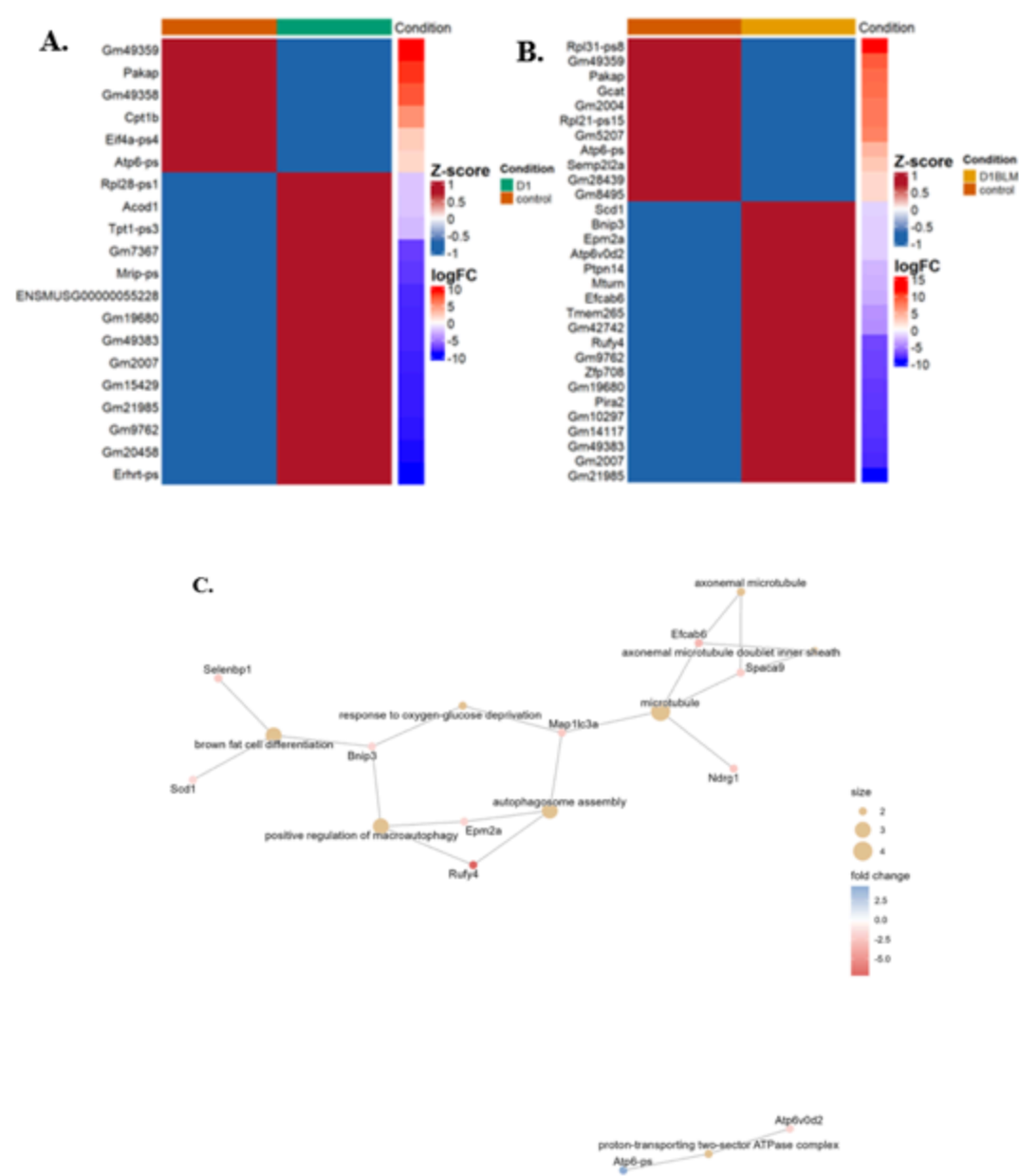


Figure 7

A. Heatmap of the top 20 differentially expressed genes in D1 Raw 264.7 macrophage cells stimulated with metabolites from *M. tuberculosis* (D1), compared to untreated control cells. **B.** Heatmap displaying the top 20 differentially expressed genes in Raw 264.7 macrophage cells stimulated with metabolites derived from *M. tuberculosis* H37Rv (D1), followed by treatment with metabolites from *B. licheniformis* OM182835 (BLM). **C.** Enriched gene ontology analysis in Raw 264.7 macrophage cells stimulated with metabolites derived from *M. tuberculosis* H37Rv (D1), followed by treatment with metabolites from *B. licheniformis* OM182835 (BLM), contrasting control versus D1BLM (D1+BLM).

Supplementary Files

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