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Biofilm formation and maturation dynamics of *Mycobacterium wolinskyi*, an emerging device-associated pathogen

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Abstract

Background: *Mycobacterium wolinskyi* is an emerging, rapidly growing non-tuberculous mycobacterium (NTM) increasingly associated with postoperative and device-related infections. Although biofilm formation is a key mechanism for the survival and persistence of NTM, the ability of *M. wolinskyi* to form biofilms has never been characterized quantitatively.

Objective: To provide the first longitudinal quantification of biofilm formation by a clinical isolate of *M. wolinskyi*, using a standardized crystal violet (CV) microtitre assay.

Methods: A wound-derived *M. wolinskyi* isolate was identified using MALDI-TOF MS, and this identification was confirmed via targeted Next Generation Sequencing (NGS). Biofilm biomass deposition and maturation were quantified longitudinally using a CV-based static microtiter model, with early short-interval measurements (48 h and 96 h) to define the onset of adhesion, followed by extended time points (weeks 1, 3, and 6) to capture structural maturation and

stabilization.

Absorbance was measured at 570 nm after dye solubilization with 30% acetic acid. Experiments were performed in technical sextuplicate and independently repeated three times. Temporal differences were evaluated using one-way ANOVA. The presence and structure of the biofilm were also assessed after one week using confocal microscopy.

Results: Biofilm quantification revealed a significant increase within the first three weeks, with optical density rising from $0,6 \pm 0,2$ to $1,9 \pm 0,3$ ($p = 0,00$). Between weeks 3 and 6, the values remained similar ($1,9 \pm 0,3$ vs. $2,0 \pm 0,3$; $p = 0,35$), indicating a plateau phase. Overall, comparing weeks one and six, confirmed a significant increase in biofilm biomass ($p = 0,00$), with stabilization occurring from week three onwards. This plateau phase indicates the consolidation of a mature, EPS-rich biofilm. According to OD interpretive criteria, the isolate transitioned from a moderate biofilm producer in week 3 to a strong producer in week six.

Conclusion: This study provides the first quantitative evidence that *M. wolinskyi* forms a mature, stable biofilm under static in vitro conditions. The observed trajectory, early formation and maturation by week three followed by stabilization by week six, align with recognized dynamics of rapidly growing NTM biofilms. It supports the pathogen's capacity for environmental persistence, antimicrobial tolerance, and device-associated infection. The standardized CV assay used in this study provides a reproducible basis for future comparative studies and for screening anti-biofilm strategies targeting emerging NTM.

Key words: *Mycobacterium wolinskyi*; *non tuberculous mycobacteria*; *biofilm formation*; *crystal violet assay*; *longitudinal quantification*; *antimicrobial tolerance*.

Introduction

Nontuberculous mycobacteria (NTM) are a diverse group of environmental organisms that are increasingly linked to human diseases, such as pulmonary infections, postoperative wound complications and contamination of medical devices [1, 2]. Among rapidly growing mycobacteria

(RGM), the ability to form biofilms is recognized as critical for environmental persistence, tolerance to antimicrobials, and colonization of abiotic surfaces in healthcare settings. Biofilms enable NTM to withstand disinfectants, survive in conditions with limited nutrients, and adopt metabolic states that significantly reduce their susceptibility to antimicrobial agents, making them extremely difficult to eradicate [3, 4, 5, 6]. In NTM species such as *Mycobacterium abscessus*, *Mycobacterium chimaera* and *Mycobacterium chelonae*, drug resistance is often linked to the presence of biofilms [7]. These biofilms contribute to the colonization of hospital plumbing systems, surgical equipment and implanted devices, thereby reinforcing the clinical relevance of biofilm-mediated persistence across RGMs [5, 6]. *Mycobacterium wolinskyi* is a recently recognized pathogen of growing clinical importance. The specific strain investigated in this study was supplied by Sant'Orsola Hospital in Bologna. This emerging RGM, associated with postoperative infections, device-related diseases, and surgical site outbreaks, remains largely understudied. Although it is being reported with increasing frequency in clinical settings [7, 8, 9, 10], its ability to form biofilms has never been quantitatively assessed. Without empirical evidence to define its biofilm phenotype, the mechanisms underlying its environmental resilience and capacity to persist on medical devices, as well as its potential for antimicrobial tolerance, remain largely undefined [4, 5]. Determining whether *M. wolinskyi* can initiate, mature, and stabilize a biofilm, and whether this trajectory resembles that of other RGM, provides essential insights into its pathogenic potential. This information can then be used to inform strategies for detection, disinfection and clinical management. Here, we present the first quantitative, longitudinal, characterization of *M. wolinskyi* biofilm formation, achieved using a standardized crystal violet (CV) microtitre assay.

The study's reproducible and transparent methodological framework is applied at defined time points (48 hours, 96 hours, one, three and six weeks). This approach will lay the groundwork for future comparative and mechanistic investigations into RGM biofilm biology. These investigations will inform the development of anti-biofilm strategies relevant to healthcare settings. Rather than aiming to resolve fine-scale kinetic phases, this study was designed to establish a reference longitudinal biofilm phenotype for an emerging and understudied RGM species, focusing on biomass deposition, matrix maturation, and stabilization under standardized static conditions.

Materials and methods

Clinical isolate and species identification

After being isolated from a wound sample (Fig. 1), the strain was identified at the Microbiology Unit of S. Orsola University Hospital in Bologna, Italy, using the MALDI Biotyper® with the MBT HT Mycobacteria Module (Bruker, Germany). To confirm identification, bacterial strains were subsequently sequenced using the Deeplex Myc-TB CE-IVD kit (GenoScreen, Lille, France) according to the manufacturer's instructions.



Figure 1. Culture plate of *Mycobacterium wolinskyi* isolate

Molecular confirmation and primary culture

After receiving the strain at our Microbiology laboratory at INMI Lazzaro Spallanzani, an Anyplex™ MTB/NTM real-time PCR detection assay was performed to rule out the presence of *Mycobacterium tuberculosis*. The strain was then cultured using the BACTEC™ MGIT™ 960 system (Becton Dickinson, Sparks, MD, USA), an automated liquid culture platform commonly used for isolating and growing mycobacteria. Following the manufacturer's instructions, the strain was inoculated into MGIT tubes containing Middlebrook 7H9 broth supplemented with OADC enrichment and PANTA antibiotic mixture. The cultures were then incubated at 37 °C in the MGIT

130 960 instrument until adequate growth was detected by the automated, fluorescence-based
131 detection system.

132

133 *Preparation of standardized inoculum*

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135 To ensure consistency across experimental replicates, actively growing MGIT cultures were
136 adjusted to a 0.5 McFarland standard. This standardized suspension was used as the inoculum for
137 biofilm formation assays in flat-bottom, non-tissue-culture-treated polystyrene 96-well plates (Cell
138 Culture Plate 96-well, SPL Life Sciences Co.). At the same time, for confocal microscopy,
139 untreated 6-well cell culture plates (Corning, USA) were prepared by inserting a sterile cover slip
140 into each well. The *M. wolinskyi* strain suspension was then applied to the coverslips to allow it to
141 adhere to the substrate, prior to the subsequent fixation and staining procedures required for
142 confocal microscopy.

143

144 *Confocal Microscopy*

145

146 Confocal microscopy was performed after one week of culture to visualize the biofilm structure.
147 Biofilms were stained using the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit and the
148 FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain (Invitrogen, Thermo Fisher Scientific, USA),
149 following the manufacturers' protocols. The LIVE/DEAD® kit enabled dual-colour fluorescence
150 assessment of bacterial viability based on cell membrane integrity, while SYPRO® Ruby provided
151 broad protein staining, including glycoproteins, phosphoproteins, lipoproteins, calcium-binding
152 proteins and fibrillar proteins. Stained samples were imaged using a Zeiss 900 LSM confocal
153 microscope equipped with an Airyscan2 detector, and the resulting image stacks were processed
154 using Zen software (Zeiss, Germany).

155

156 *Biofilm formation assay*

157

158 Biofilm formation was assessed using a longitudinal adaptation of the crystal violet (CV) microtiter
159 plate assay. For each biological replicate, six wells of a flat-bottom, non-tissue-culture-treated
160 polystyrene 96-well plate were inoculated with the standardized bacterial suspension, while six

161 wells containing sterile Middlebrook 7H9 broth served as negative controls. Plates were incubated
162 under static conditions at 37 °C in an atmosphere enriched with 5% CO₂, using a sterile plastic lid
163 to minimize evaporation and ensure a stable microenvironment throughout biofilm development.
164

165 Biofilm development was evaluated at 48 hours, 96 hours, one week, three weeks, and six weeks.
166 Short time intervals during the first week (48 h and 96 h) were included to verify the absence of
167 detectable adhered biomass at early time points. Subsequent time points were intentionally
168 spaced to capture progressive extracellular matrix deposition and architectural maturation, rather
169 than rapid biomass accumulation. This longitudinal design reflects the slow consolidation
170 dynamics of mycobacterial biofilms under static conditions and was chosen to characterize biofilm
171 stabilization rather than fine-scale growth kinetics.

172 At each time point, the culture supernatant was gently removed, and wells were rinsed with 150
173 µL of phosphate-buffered saline (PBS) to eliminate non-adherent material while preserving
174 adhered biomass. If crystalline residues were observed, the washing step was repeated. Biofilms
175 were then fixed with 150 µL of cold methanol for 20 minutes to stabilize cellular and extracellular
176 matrix components, followed by air drying under a laminar flow hood.
177

178 As a positive control, the same protocol was applied to the well-established biofilm-producing
179 strain *Pseudomonas aeruginosa* ATCC 29853. Due to its faster biofilm-development dynamics,
180 measurements were performed after 24 hours.
181

182 *Crystal violet staining and biomass quantification* 183

184 The fixed biofilms were stained with 100 µL of 0.1% (w/v) crystal violet solution for 30 minutes at
185 room temperature. Crystal violet is a cationic dye that binds to the negatively charged
186 components of the biofilm matrix, such as nucleic acids, polysaccharides and proteins. This
187 enables the total biomass to be visualized and quantified. The intensity of the stain correlates with
188 the amount of biofilm present. Excess stain was removed, and the wells were rinsed repeatedly
189 with 100 µL of PBS until the wash was clear. The plates were then dried overnight before the
190 bound dye was solubilized with 150 µL of 30% glacial acetic acid for 15 minutes to release the
191 crystal violet for spectrophotometric quantification. One hundred microlitres of the solubilized dye

192 were transferred to a new microplate after which the absorbance was measured at 570 nm using a
193 microplate spectrophotometer (BioTek Synergy HTX Multimode Reader, Agilent Technologies,
194 USA).

195

196 *Experimental design and reproducibility*

197

198 Five independent biological experiments were performed, each comprising six technical replicates
199 per condition. To ensure accurate biomass quantification, background absorbance from negative
200 control wells was subtracted from the corresponding experimental readings.

201

202 *Statistical analysis*

203

204 Statistical analyses were performed using the mean OD₅₇₀ value from six technical replicates of
205 five independent experiments at each time point (48h, 96h, one, three and six weeks). Changes in
206 biofilm biomass over time were assessed using a one-way ANOVA with time as the independent
207 variable. To distinguish the initial growth phase from the subsequent stabilization phase, the
208 change in OD₅₇₀ between 48 hours, 96 hours, weeks one, three and six was calculated for each
209 experiment, followed by Tukey's post hoc test for multiple comparisons. A significance threshold
210 of $p < 0.05$ was applied.

211

212 **Results**

213

214 *Species confirmation and culture characteristics*

215

216 Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry analysis using the
217 MALDI Biotyper Mycobacteria Library allowed the species to be identified as *Mycobacterium*
218 *wolinskyi* with a high score (>1.8). The result obtained by hsp65-based sequencing using the
219 Deeplex Myc-TB test confirmed the identity of the bacterial isolates as *M. wolinskyi*. The isolate
220 grew within the expected timeframe for rapidly growing non-tuberculous mycobacteria (NTM) in
221 the BACTEC™ MGIT™ 960 system (Becton Dickinson), and Ziehl–Neelsen staining revealed
222 abundant acid-fast bacilli with typical morphology.

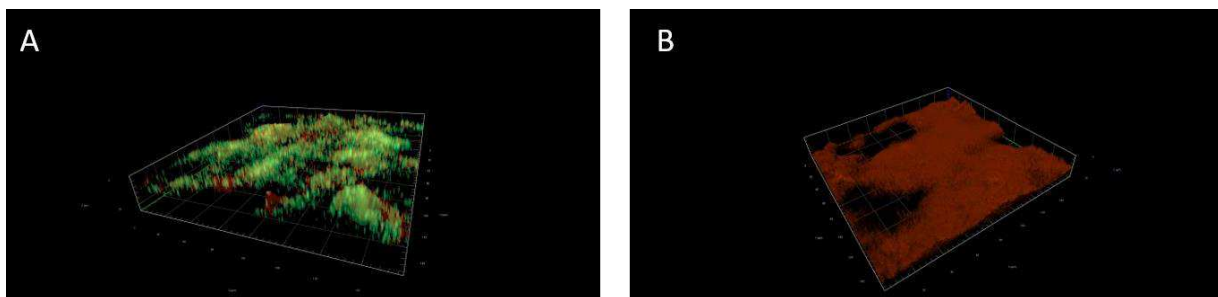
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224 *Confocal Microscopy*

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226 Confocal laser scanning microscopy performed after one week of culture confirmed the presence
227 of an extracellular matrix associated with the bacterial colonies. Staining with the FilmTracer™
228 LIVE/DEAD® Biofilm Viability Kit revealed the spatial distribution of viable and membrane-
229 compromised cells within the biofilm (Fig. 2A). Meanwhile, FilmTracer™ SYPRO® Ruby staining
230 highlighted the protein components of the extracellular matrix surrounding and interconnecting
231 the bacterial aggregates (Fig. 2B).

232



233

234 **Figure 2** Confocal microscopy images showing (A) the LIVE/DEAD assay, which distinguishes
235 cells with intact membranes (green) from those with compromised membranes (red), and (B)
236 SYPRO® Ruby staining, used to visualize the protein component of the biofilm matrix. Confocal
237 microscopy was used for qualitative structural assessment.

238

239

240 *Longitudinal quantification of biofilm formation*

241

242 In the five independent experiments, a first measurement was taken at 48 hours and a second at
243 96 hours to assess whether measurable adhered biomass was already detectable at early time
244 points. These early measurements confirmed the absence of measurable adhered biomass,
245 thereby defining week one as the earliest time point at which biofilm deposition became
246 detectable in this model.

247 These first two measurements yielded OD values of $0.2 (\pm 0.4)$ and $0.2 (\pm 0.1)$, respectively.

248 The biofilm biomass increased between the first and third weeks, stabilizing in the sixth week. The
249 average OD₅₇₀ ($\pm$ SD) values were $0.6 (\pm 0.2)$ in the first week, $1.9 (\pm 0.3)$ in the third week and

250 2.0 (± 0.2) in the sixth week. A significant increase in biofilm biomass ($p < 0.05$) was observed only
251 between the first and third week, and between the first and sixth week. By contrast, the increase
252 recorded between the third and sixth week was not significant ($p > 0.05$). The lack of significant
253 variation between weeks three and six suggests that major architectural consolidation had already
254 occurred by week three, supporting the use of this time point as a marker of biofilm maturation
255 rather than indicating insufficient temporal resolution.

256 The protocol was also applied to the *P. aeruginosa* ATCC 29853 strain, a well-known biofilm
257 producer. As before, OD values were corrected by subtracting the OD of the controls. The strain
258 showed a clear ability to form biofilm, with an average reading of 1.9 (± 0.1). The biofilm
259 measurements for *M. wolinskyi* are consistent with a transition from an initially heterogeneous
260 attachment phase to a more mature and homogeneous biofilm structure. It is also important to
261 note that crystal violet staining quantifies the total amount of biomass that has adhered, without
262 distinguishing between viable and non-viable cells. Therefore, the observed stabilization reflects
263 architectural maturation rather than metabolic activity. In static assays, rapidly growing
264 mycobacteria often require an extended incubation period for EPS consolidation and community
265 homogenization. The plateau observed between weeks three and six is thus consistent with the
266 development of a stabilized matrix, as reported for other RGMs under comparable conditions.

267

268 *Biofilm producer classification*

269

270 Based on standard OD interpretative criteria, the isolate exhibited a weak-to-moderate biofilm
271 phenotype from the first to the third week (Fig. 3), with a tendency towards strong biofilm
272 production by the sixth week. Since the quantified total adhered biomass does not distinguish
273 between viable and non-viable cells, the observed stabilisation is likely to reflect architectural
274 maturation rather than metabolic arrest. These results suggest that *M. wolinskyi* initiates biofilm
275 formation and develops into a robust, persistent producer over prolonged incubation. This is
276 consistent with other rapidly growing non-tuberculous mycobacteria (NTM) that can form stable
277 extracellular polymeric structures [5, 6].

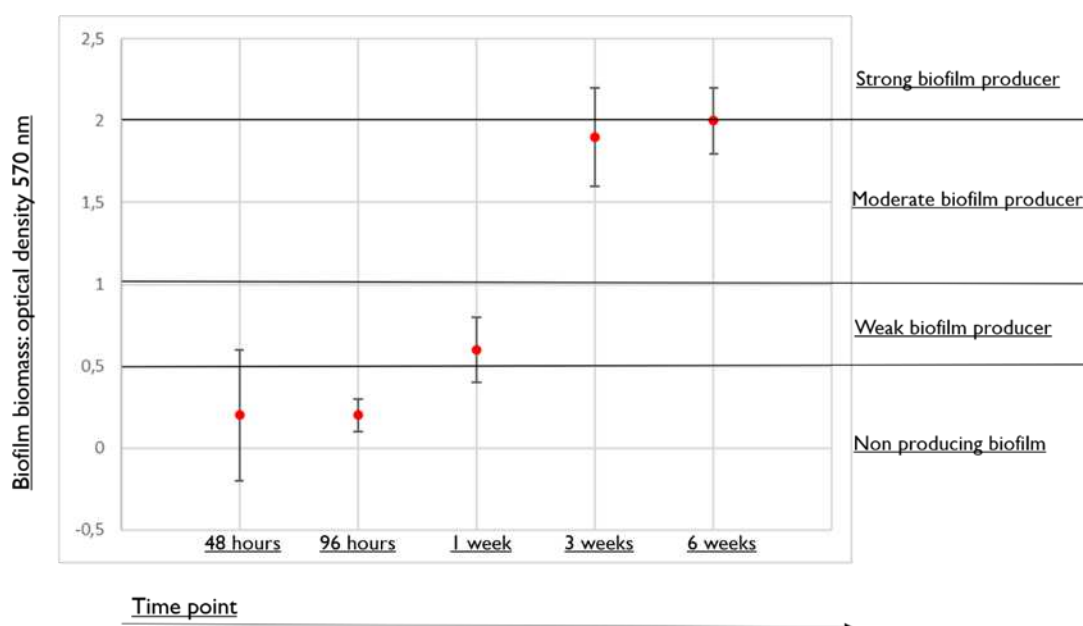


Figure 3. Biofilm biomass of *M. wolinskyi* over time under static conditions. Mean OD₅₇₀ values (± SD) from crystal violet staining at 48h, 96h, one, three, and six weeks are shown. Measurements were obtained from five independent experiments, each performed in technical sextuplicates 48 hours: 0.2 ± 0.4 ; 96 hours 0.2 ± 0.1 ; Week 1: 0.6 ± 0.2 ; Week 3: 1.9 ± 0.3 ; Week 6: 2.0 ± 0.2 .

Reproducibility of the assay

The temporal trends in OD, were reproducible across the five biological replicates showing consistent patterns of increase followed by stabilization. The negative control wells remained consistently low, validating the specificity of crystal violet binding to true adherent biomass. Furthermore, the inclusion of the biofilm-producing strain *Pseudomonas aeruginosa* ATCC 29853 provided additional confirmation of the reliability of the method.

Discussion

This study provides the first longitudinal, quantitative demonstration that *M. wolinskyi* can form a structured, progressively maturing biofilm in a controlled in vitro environment. By applying a standardised CV microtitre assay to a clinical isolate, we addressed a critical knowledge gap regarding the biofilm-forming capacity of this emerging, RGM. Despite an increasing number of clinical reports, the ability of this bacterium to persist on abiotic surfaces has remained largely uncharacterized [4, 5, 8, 9, 10]. The primary objective of this study was to establish whether *M.*

wolinskyi is capable of forming a stable and mature biofilm under static in vitro conditions, rather than to dissect fine-scale temporal kinetics. Within this framework, the observed trajectory, early biomass deposition followed by stabilization, represents a characteristic pattern of mycobacterial biofilm maturation. The six-week incubation period was intentionally extended to capture late-stage architectural consolidation, a phase that is often overlooked in short-term assays. In the context of an understudied and emerging species such as *M. wolinskyi*, the use of a single, well-characterized clinical isolate represents a deliberate and appropriate first step to establish a reference biofilm phenotype before broader inter-strain comparisons. However, detailed temporal trajectories have not been systematically characterized for rapidly growing mycobacteria. In static assays, rapidly growing mycobacteria often require prolonged incubation to consolidate EPS and homogenize the community. Preliminary attempts using standard CV protocols, commonly applied to non-mycobacterial species, failed to generate detectable biomass in *M. wolinskyi*, even in the presence of positive (data not shown). This indicates that NTM biofilm architecture requires specific fixation and staining conditions, supporting the need for the adapted CV workflow implemented in this study. The protocol described here enabled us to obtain solid and reproducible results in our study on biofilm formation by NTM microorganisms. The reliability of the method was further confirmed by the inclusion of the *Pseudomonas aeruginosa* ATCC 29853 strain, known for its ability to produce biofilm, as a positive control. Analysis of the data at different time points allowed us to characterize the initial stages of extracellular matrix deposition during the first week of culture, as well as the subsequent stages of biofilm maturation. Interestingly, measurements taken at 48 and 96 hours revealed no detectable biomass, suggesting that the cells remained predominantly planktonic during the initial phase. Subsequent time points, on the other hand, showed a transition from an initially heterogeneous adhesion phase to a more mature, stable and homogeneous biofilm structure. This interpretation aligns with established models of mycobacterial biofilm maturation. It is also important to note that crystal violet staining quantifies total adhered biomass without distinguishing between viable and non-viable cells. Therefore, the observed stabilization reflects architectural maturation rather than metabolic activity. In static assays, rapidly growing mycobacteria often require prolonged incubation for EPS consolidation and community homogenization. The plateau observed between weeks three and six is consistent with the development of a stabilised matrix, as has been described for other RGMs under comparable conditions. Notably, the marked reduction in

331 variability between replicates from 48 hours to week six suggests a shift from an early-stage
332 heterogeneous community to a more uniform EPS matrix [11]. This matrix consolidation is a
333 hallmark of structural maturation, typically involving increased resilience and reduced metabolic
334 heterogeneity. In this context, the plateau in OD indicates structural consolidation within the
335 matrix rather than cessation of biological activity. This is consistent with the formation of a robust,
336 EPS-rich *M. wolinskyi* biofilm. From clinical and environmental perspectives, *M. wolinskyi*'s
337 ability to form mature, persistent biofilms provides a plausible mechanistic basis for its association
338 with postoperative and device-related infections.

339 Biofilm-embedded mycobacteria are known to exhibit significantly increased antimicrobial
340 tolerance compared to their planktonic counterparts due to restricted drug penetration, altered
341 growth states and up-regulation of efflux pumps [4, 7, 12, 13]. The documented phenotype
342 strengthens the hypothesis that the environmental or device persistence of *M. wolinskyi* may be
343 mediated by biofilms. This emphasizes the importance of improved detection methods, more
344 rigorous decontamination procedures and the consideration of anti-biofilm strategies, including
345 the use of biofilm-disrupting agents and, when necessary, the removal of the device, in clinical
346 management [8, 9, 10]. Beyond biological characterization, this work contributes to
347 methodological standardization. In order to harmonize CV-based quantification and facilitate
348 inter-laboratory comparisons, we have explicitly documented the following key experimental
349 parameters: fixation and staining steps, solvent concentrations, absorbance wavelength, and
350 incubation conditions (static model at 37 °C, with 5% CO₂ enrichment and sterile plastic plate lid).
351 This is in line with recent calls for protocol transparency in NTM biofilm assays [14, 15]. These
352 standardized conditions provide a reproducible platform for the longitudinal measurement and
353 subsequent screening of anti-biofilm interventions.

354

355 **Limitations**

356

357 This study examined a single clinical isolate in controlled static conditions. Biofilm development
358 under flow, nutrient limitation and host-mimicking microenvironments was not assessed. CV
359 staining quantifies total adhered biomass and cannot distinguish between viable and non-viable
360 cells. It also cannot determine the composition or spatial architecture of EPS. Therefore, OD
361 stabilization reflects structural consolidation rather than metabolic quiescence. Future work

362 should incorporate multiple clinical and environmental isolates and EPS compositional profiling to
363 gain a deeper mechanistic understanding.

364

365 **Future Perspectives**

366

367 Building on these findings, future work will expand the isolate panel to include additional clinically
368 relevant and frequently encountered NTM species, particularly *Mycobacterium abscessus* and
369 other rapid growers. This broader comparative framework will allow us to determine whether the
370 biofilm-development patterns observed here are conserved across pathogenic NTM lineages and
371 to delineate species-specific traits that may influence biofilm maturation, persistence, and
372 environmental resilience.

373

374 **Conclusion**

375

376 In summary, *M. wolinskyi* forms a mature, EPS-rich biofilm within six weeks, after which the
377 structure stabilizes. This behaviour enables it to persist in healthcare environments and contribute
378 to device-associated infections. By integrating longitudinal quantification with a standardised
379 protocol, this study provides a reproducible framework for comparative and mechanistic research
380 on NTM biofilms, thereby facilitating the development of targeted anti-biofilm strategies. The
381 reliability of this approach for NTM is demonstrated by previous protocols in which a biofilm-
382 producing control strain, such as *P. aeruginosa*, was used but failed to generate positive results
383 (data not shown). The need to optimize a NTM-specific crystal violet protocol further suggests that
384 their biofilm architecture differs substantially from that of other bacteria.

385

386 **Declarations**

387

388 **Abbreviations**

389

390 **NTM** – Nontuberculous Mycobacteria

391 **RGM** – Rapidly Growing Mycobacteria

392 **CV** – Crystal Violet

393 **EPS** – Extracellular Polymeric Substances
394 **INMI** - Istituto Nazionale per le Malattie Infettive
395 **OD / OD₅₇₀** – Optical Density at 570 nm
396 **PBS** – Phosphate-Buffered Saline
397 **MALDI-TOF MS** – Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass
398 Spectrometry
399 **NGS** – Next Generation Sequencing
400 **PCR** – Polymerase Chain Reaction
401 **MGIT** – Mycobacteria Growth Indicator Tube
402 **OADC** – Oleic acid–Albumin–Dextrose–Catalase supplement
403 **PANTA** – Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin
404 **LSM** – Laser Scanning Microscope
405 **MBT HT** – MALDI Biotyper High-Throughput Mycobacteria Module (Bruker)
406 **CE-IVD** – Conformité Européenne – In Vitro Diagnostic (Deplex Myc-TB kit)
407 **LIVE/DEAD®** – FilmTracer LIVE/DEAD Biofilm Viability Kit
408 **SYPRO® Ruby** – FilmTracer SYPRO Ruby Biofilm Matrix Stain
409 **ATCC** – American Type Culture Collection
410 **w/v** — weight/volume
411 **ANOVA** – Analysis of Variance
412 **SD** – Standard Deviation
413 **IRCCS** – Istituto di Ricovero e Cura a Carattere Scientifico
414 **GDPR** – General Data Protection Regulation
415 **MIRRI-IT** – Microbial Resource Research Infrastructure – Italian Node
416

417 **Ethics approval and consent to participate**

418 This study did not involve human participants, human data, human tissue, or human specimens. It
419 used exclusively anonymized bacterial isolates obtained during routine diagnostic activity at the
420 National Institute for Infectious Diseases “Lazzaro Spallanzani” IRCCS. According to Italian
421 Legislative Decree 196/2003 (Art. 110-bis) and Regulation (EU) 2016/679 (GDPR), IRCCS
422 institutions may process anonymized biological materials collected during healthcare activities for

research purposes consistent with their institutional mission without requiring Ethics Committee approval. For this reason, the INMI Ethics Committee confirmed that formal approval was not required for studies based solely on anonymized microbial isolates. All procedures were performed in accordance with relevant institutional guidelines and national regulations. Consent to participate was not required because no human participants or identifiable human data were involved. Consent for publication: not applicable.

Availability of data and materials

All datasets generated and analyzed during the current study are included within the published article. No raw sequencing data or publicly depositable molecular datasets were produced. Species identification was performed using the commercial Deeplex MycTB CE-IVD assay, which does not generate publicly accessible raw sequencing files.

Competing interest

The authors declare that they have no competing interest.

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Author Contributions:

conceptualization, methodology, writing—original draft preparation V.D.;
data curation, investigation, methodology I.P.;
investigation, review and editing P.D.M., F.B., L.F.;
investigation A.C.;
writing—review and editing, data curation C.N., R.G;
project administration, funding acquisition, writing—review and editing C.F.
All authors have read and agreed to the published version of the manuscript.

Clinical trial number: not applicable.

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