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1 **Investigation of skin microbiota reveals *Mycobacterium ulcerans*-*Aspergillus* sp. trans-**
2 **kingdom communication**
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21 **ABSTRACT**

22 **Background**

23 *Mycobacterium ulcerans* secrete a series of non-ribosomal-encoded toxins known as mycolactones that
24 are responsible for causing a disabling ulceration of the skin and subcutaneous tissues named Buruli
25 ulcer. The disease is the sole non-contagion among the three most common mycobacterial diseases in
26 humans. Direct contact with contaminated wetlands is a risk factor for Buruli ulcer, responsible for *M.*
27 *ulcerans* skin carriage before transcutaneous inoculation with this opportunistic pathogen.

28 **Methodology and principal findings**

29 In this study, we analysed the bacterial and fungal skin microbiota in individuals exposed to *M. ulcerans*
30 in Burkina Faso. We showed that *M. ulcerans*-specific DNA sequences were detected on the unbreached
31 skin of 6/52 (11.5%) asymptomatic farmers living in Sindou versus 0/52 (0%) of those living in the non-
32 endemic region of Tenkodogo. Then, we cultured the skin microbiota of asymptomatic *M. ulcerans*
33 carriers and negative control individuals, all living in the region of Sindou. A total of 84 different
34 bacterial and fungal species were isolated, 21 from *M. ulcerans*-negative skin samples, 31 from *M.*
35 *ulcerans*-positive samples and 32 from both. More specifically, Actinobacteria, *Aspergillus niger* and
36 *Aspergillus flavus* were significantly associated with *M. ulcerans* skin carriage. We further observed that
37 *in vitro*, mycolactones induced spore germination of *A. flavus*, attracting the fungal network.

38 **Conclusion**

39 These unprecedented observations suggest that interactions with fungi may modulate the outcome of *M.*
40 *ulcerans* skin carriage, opening new venues to the understanding of Buruli ulcer pathology, prophylaxis
41 and treatment of this still neglected tropical infection.

42

43 **Keywords:** *Aspergillus* spp., *Aspergillus niger*, *Aspergillus flavus*, Buruli ulcer, *Mycobacterium*
44 *ulcerans,* skin microbiota, fungi, *Penicillium* spp., *Penicillium rubens.*

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45 **Author summary**

46 Buruli ulcer is a chronic infectious disease caused by the environmental opportunistic pathogen
47 *Mycobacterium ulcerans* which secretes an exotoxin responsible for its pathogenicity. The reservoir and
48 sources of *M. ulcerans* in the environment remain elusive and its mode of transmission is unclear. To
49 acquire *M. ulcerans* infection, at least two conditions must be met, viable bacteria and a skin lesion as
50 demonstrated by experimental animal models. In this study, we showed that *M. ulcerans* specific DNA
51 sequences could be detected on the healthy skin of asymptomatic farmers living in one region of Burkina
52 Faso where Buruli ulcer cases had already been reported, but not in Buruli ulcer-free regions, suggesting
53 skin carriage after contacts with environmental sources. We also investigated the skin microbiota of *M.*
54 *ulcerans* carriers and found significant associations of some bacteria and fungi with skin carriage of *M.*
55 *ulcerans.* These associations may due to the effect of mycolactones on some fungi species. As we
56 showed previously with *Mucor circinelloides* and here with *Aspergillus flavus.*

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57 **Introduction**

58 Buruli ulcer is a disabling chronic disease of the skin and the subcutaneous tissues [1].
59 The disease has been reported since 1948 in several tropical regions [2], with the highest incidence being
60 observed in West African countries, including Côte d'Ivoire, Ghana, and Benin [3]. The causative agent
61 *Mycobacterium ulcerans* is a non-tuberculous mycobacterium harbouring a 174-kb circular virulence
62 plasmid pMUM [4-5] encoding three genes, *mls*A1, *mls*A2 and *mls*B, responsible for the non-ribosomal
63 synthesis of mycolactone toxins, the main virulence factors of the pathogen [6]. This macrolide exotoxin
64 is secreted by a group of closely related non-tuberculous mycobacteria named Mycolactone Producing
65 Mycobacteria (MPM) [7-8]. Comparative analysis of whole genome sequences showed that MPM form a
66 single clonal group that evolved from a *Mycobacterium marinum* parent [9]. This group is divided into
67 three lineages, including frog and fish pathogens in one lineage [10-11], the Japanese strain *M. ulcerans*
68 subsp. *shinshuense* in a second lineage*,* while the third lineage includes a highly clonal group responsible
69 for Buruli ulcer in Africa and Australia [9]. Every strain synthesizes at least one of the eight congeners
70 of mycolactone A/B named mycolactone C, D, E, F, dia-F, S1 and S2 [12]. Each type of mycolactone
71 exhibits a variable degree of cytotoxicity, with mycolactone A/B being the most biologically active [13].
72 Indeed, this macrolide toxin is responsible for cytotoxic effects, namely, apoptosis and necrosis, in
73 addition to immunosuppressive and analgesic effects [12] after *M. ulcerans* has penetrated breached skin
74 to initiate discrete oedematous lesions that can evolve into typical Buruli ulcer lesions [14]. Currently,
75 the mode of transmission of *M. ulcerans* is debated. While it is clear that *M. ulcerans* must be inoculated
76 through the skin to elicit Buruli ulcer, the specific role of aquatic bugs and mosquitoes as potential
77 vectors is debated along with the possibility of passive entry by any skin breach regardless of its cause. It
78 is not known whether *M. ulcerans* can colonize skin in asymptomatic populations exposed to
79 environments contaminated with *M. ulcerans*. Mycobacteria of the *M. ulcerans* group are known as
80 environmental organisms residing in poorly defined aquatic ecological niches where they could be part

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81 of an alimentary chain [15]. We previously reported that *M. ulcerans* could thrive in environmental
82 niches containing bacteria, fungi, algae and mollusks with which *M. ulcerans* may exchange nutrients
83 [16]. One may hypothesize that the cutaneous microbiota, which is part of the individual, partially
84 controls the expression of the *M. ulcerans* infection [17]. Indeed, bacteria and fungi present on skin
85 contaminated with *M. ulcerans* could interact with the pathogen, as previously shown with the
86 antagonism between *Staphylococcus lugdunensis* and *Staphylococcus aureus* in the nasal mucosa [18-
87 19].
88 To contribute to this medical debate, we have undertaken the first study to evaluate the possibility of
89 asymptomatic carriage of *M. ulcerans* on healthy skin, to characterize the cutaneous bacterial and fungal
90 microbiota associated with the asymptomatic carriage of *M. ulcerans* and to assess the biological
91 interactions between *M. ulcerans* and the skin microbiota.
92

93 **Methods**

94 **Ethics Statement**

95 This study was approved by the Centre MURAZ Ethics Committee, Burkina Faso, and reference
96 2018- 11/MS/SG/CM/CEI.
97 All adult subjects provided informed oral consent, and a parent or guardian of any child
98 participant provided informed oral consent on the child's behalf after explaining the merits of the
99 study. Written consent could not be obtained because the study was conducted among an illiterate
100 population. The research presents no more than minimal risk of harm to subjects and involves no
101 procedures for which written consent is normally required outside of the research context (Sec.
102 56.109 IRB review of research).
103 **Sample collection.** Sampling was performed in two regions of Burkina Faso. Sindou is located in
104 the rural district of Niofila, Douna Department, Province of Léraba in the Cascades region in the

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105 southwest of Burkina Faso near the borders with Côte d'Ivoire and Mali. People sampled in this
106 region are farmers in frequent contact with stagnant water because of their daily activities in rice,
107 banana and vegetable cultivation areas that are irrigated from a nearby dam. Cases of Buruli ulcer
108 have already been reported in this region [20]. Tenkodogo, a town located in the province of
109 Boulgou and the Central-Eastern region of Burkina Faso, was used as a negative control region
110 free of Buruli ulcer. Additionally, in this region, farmers working near two dams in rice and
111 vegetable cultures were swabbed (the lower legs of the farmers were cleaned with water and then
112 swabbed with sterile swabs (Deltalab, Barcelona, Spain) containing 1 mL of sterile 0.9% sodium
113 chloride solution, the swabbed parts did not contain any visible lesions.). A total of 104 farmers
114 were sampled, 52 farmers in each region. In Sindou region, the average age was 37 years, 30
115 females and 22 males. In Tenkodogo region the male/female ratio was 27/25 respectively with an
116 average age of 20 years. One sample was taken per individual.

117 **Detection of *M. ulcerans* DNA.**

118 **Real-time PCR amplifications.** Total DNA from the samples and from a six-week-old culture of
119 *M. ulcerans* CU001 (positive control) was extracted using a QIAamp Tissue Kit by QUIAGEN-
120 BioRobot EZ1, according to the manufacturer's instructions (Qiagen, Hilden, Germany). To
121 assess PCR inhibition, 10 µL of an external control was added to 190 µL of sample volume as
122 previously described [21. Extracted DNA was used in real-time PCR (RT-PCR) to amplify the
123 insertion sequences (IS*2404* and IS*2606*) and the ketoreductase-B domain of the mycolactone
124 polyketide synthase genes (KR-B) [22] using RT-PCR reagents from Roche PCR Kit (Roche
125 Diagnostics, Meylan, France) and primers and probes as previously described [21] in a CFX 96™
126 real-time PCR thermocycler and detection system (BIO-Rad, Marnes-la-Coquette, France). To
127 estimate *M. ulcerans* inoculum in skin swabs, we performed three calibration curves for the
128 IS*2404*, IS*2606*, and KR-B genes. The total DNA of the *M. ulcerans* CU001 strain calibrated at 1

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129 McFarland = 10^6 CFU was extracted with EZ (Qiagen, Hilden, Germany). Then, 10-fold serial
130 dilutions of up to 10^{-8} were made to generate a calibration curve for each system. Two reactional
131 mixes were incorporated into each PCR run as negative controls. Samples were considered
132 positive when the KR-B gene was detected with Ct $<$ 40 cycles, and at least one of the two PCRs
133 of the insertion sequence gave Ct $<$ 40 cycles and KR-B was detected with Ct $<$ 40 cycles.

134 **Skin microbiota repertoire.**

135 **Bacterial culture.** All RT-PCR-positive samples collected in Sindou were selected for culture. Six
136 negative samples collected in the same region were used as negative controls. For each sample, a cascade
137 dilution (up to 10^{-10}) was performed in sterile PBS (Thermo Fisher Diagnostics, Dardilly, France), and
138 100 µL of each dilution was inoculated in duplicate on blood agar plates (bioMérieux, Marcy l'Etoile,
139 France). One blood agar plate was incubated aerobically, and the second was anaerobically incubated at
140 37°C for 72 hours. After incubation, each colony presenting a unique morphology was sub-cultured onto
141 a blood agar plate and incubated at 37°C for 48 hours under the appropriate atmosphere to isolate each
142 colony type.

143 **Fungal culture.** *M. ulcerans* PCR-positive samples were cultured in three different culture media,
144 namely, homemade RMI medium, Sabouraud agar (Oxoid, Dardilly, France) and Chromagar (Becton
145 Dickinson, Le Pont de Claix, France) incubated at 30°C for seven days. Each colony was sub-cultured
146 and incubated under the same conditions. The six PCR-negative samples (negative controls) were treated
147 using the same protocol.

148 **Matrix assisted laser desorption ionization time-of-flight identification of colonies.** The
149 identification of the bacterial and fungal colonies was carried out using matrix-assisted laser
150 desorption ionization time-of-flight mass spectrometry MALDI-TOF-MS as previously described
151 [23,24]. For bacteria, each colony was deposited in duplicate onto a MALDI-TOF MSP 96 target
152 plate (Bruker Daltonics, Leipzig, Germany), and 2 µL of matrix solution (saturated solution of

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153 alpha-cyano-4- hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was
154 added to each spot and allowed to dry for 5 mins and then analysed by Microflex spectrometer
155 (Bruker Daltonics) using the software MALDI BioTyper 3.0 (Bruker Daltonics). For the
156 identification of fungi, each colony was incubated in 1 mL of 70% ethanol for 10 min and then
157 centrifuged at 1,300 g for 5 min. The pellet was treated with 20 µL of acetonitrile and formic acid
158 (v.v) at 100% and 70%, respectively. This mixture was then centrifuged at 1300 g for 5 min, and
159 1.5 µL of the supernatant was deposited on a MALDI-TOF-MS target and allowed to dry before
160 1.5 µL of matrix was deposited on each spot, allowed to dry for 5 min and then analysed by par
161 Microflex spectrometer (Bruker Daltonics) using the software MALDI BioTyper 3.0 (Bruker
162 Daltonics).
163 **Molecular identification and sequencing.** All colonies that remained unidentified by MALDI-TOF-MS
164 were subjected to molecular identification by sequencing the bacterial 16S rRNA [25] and the fungal
165 ITS1, ITS2, β tubulin and TEF regions. The primers used in this study are reported in Table S1. DNA
166 extraction was performed using BioRobot EZ1 (Qiagen, Les Ullis, France) using the commercial EZ1
167 DNA Tissue Kit according to the manufacturer's instructions (Qiagen). PCR was performed using
168 Hotstar *Taq* polymerase according to the manufacturer's instructions (Qiagen) using a thermocycler
169 (Applied Biosystem, Paris, France). PCR products were separated by electrophoresis on a 1.5% agarose
170 gel and stained with SYBR® safe (Thermo Fisher Scientific) before being visualized under an ultraviolet
171 transilluminator. PCR products were then purified using a Millipore NucleoFast 96 PCR kit following
172 the manufacturer's recommendations (Macherey-Nagel, Düren, Germany) and sequenced using the
173 BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with an automatic sequencer ABI
174 (Applied Biosystems). Sequences were assembled using the software ChromasPro 1.7 (Technelysium
175 Pty Ltd., Tewantin, Australia) and blasted in the NCBI databank (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

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176 to identify bacterial species and against the Mycobank database http://www.mycobank.org/ to identify
177 fungal species.

178 **Fungi-*M. ulcerans* biological interactions.** The three fungal species found to significantly correlate
179 with the presence / absence of *M. ulcerans* skin carriage (*A. flavus*, *A. niger* and *P. rubens*) were cultured
180 in the presence / absence of mycolactones AB / C extracted from a culture of *M. ulcerans* CU001 to
181 observe the effect of mycolactones on spore germination and the fungal network. Briefly, agar plates
182 were cut into T-shape, the fungal spores were placed on the middle strand, and two virgin absorbent
183 paper discs were placed on each side of the plates. 20uL of PBS were put on the first disc (negative
184 control) and 20uL of mycolactones AB / C on the second disc. The chemoattractant effect of
185 mycolactone AB / C was measured as previously described [25]. On the other hand, fungal spores were
186 put in Sabouraud liquid medium containing 20uL of mycolactones AB / C or 20uL of PBS as a negative
187 control. The effect of Mycolactone AB/C on spore germination was then monitored as previously
188 described [26].

189 **Statistical analyses.**

190 The first exploratory step of unsupervised analysis of the cutaneous bacterial and fungal
191 microbiota used a main component analysis by integrating the presence or absence of each of the
192 fungi detected in at least one individual and adding the variable "detection of *M. ulcerans* by
193 PCR". We complemented this analysis with a supervised comparison between individuals
194 positive and negative for *M. ulcerans* by PCR. To do this, we compared the detection frequency
195 in the two groups. Statistical significance was calculated by the two-sided exact Fisher's test.
196 Owing to the possible risk of missing important findings, adjustments for multiple comparisons
197 were not performed, as suggested for exploratory work [27]. A double-clustering heatmap was
198 used to visualize the potential clustering of cultured fungal and bacterial repertoires with *M.*
199 *ulcerans*-positive or negative skin samples. Finally, we carried out a discriminant factor analysis

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200 to test whether the fungal skin microbiota discriminated against individuals who were positive or
201 negative for *M. ulcerans* skin carriage. The principal component and discriminant factor analyses
202 were performed using XLSTAT v2019.1 statistical and data analysis solution (Long Island, NY,
203 USA (https://www.xlstat.com)). Multivariate analysis was performed via the Genmod procedure
204 with the SAS 9.4 statistical software using a negative binomial distribution, adjusted for the
205 effect of time (quantitative variable) and by using generalized estimating equations to account for
206 the non-independence of repeated measures to analyse the results of germination assays. The
207 Wilcoxon signed rank test and Chi-squared test were used to compare the attraction effect of
208 fungi by mycolactones and to compare *M. ulcerans* prevalence in the two geographical regions of
209 Sindou and Tenkodogo, respectively. Statistical tests were performed on the website biostaTGV,
210 https://biostatgv.sentiweb.fr.

211 **Results**

212 ***M. ulcerans* DNA detection.** Negative controls introduced in every batch of real-time PCR remained
213 negative, and positive controls were positive for all the targeted sequences IS*2404*, IS*2606* and KR-B,
214 allowing the establishment of calibration curves. Of the 52 swabs collected in the region of Sindou, eight
215 were positive for IS*2404*, six were positive for IS*2606*, two were positive for both IS*2404* and IS*2606*,
216 one was positive for both IS*2404* and KR-B, two were positive for both IS*2606* and KR-B, one was
217 positive for all three targeted sequences and 32 samples were negative (Fig. 1). Therefore, 6/52 (11.5%)
218 swabs were positive for at least two *M. ulcerans* DNA sequences. As expected, only three samples were
219 positive for IS*2404* only in Tenkodogo, so none of the 52 skin samples collected in this negative control
220 region were positive for *M. ulcerans,* confirming the probable absence of *M. ulcerans* in this region. The
221 difference in the prevalence of RT-PCR-based detection of *M. ulcerans* between the two regions was

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222 significant (P = 0.04, N-1 Chi-squared test). Based on the calibration curves established during this
223 study, the Ct values observed here were extrapolated to 9-50 colony forming units (CFU) of *M. ulcerans*.
224 **Skin microbiota identification**. A total of 84 different species of microorganisms (62 bacterial and 22
225 fungal species) belonging to 45 different genera were recovered from the twelve samples. Thirty-one
226 different species of microorganisms (19 bacterial and 12 fungal species) grew exclusively in *M.*
227 *ulcerans*-PCR positive samples. Thirty-two different species (22 bacterial and 10 fungal species) grew
228 only from *M. ulcerans*-PCR negative samples. Twenty-one bacterial species grew on both *M. ulcerans*-
229 PCR positive samples and *M. ulcerans*-PCR negative samples. No fungal species were found in both
230 types of samples simultaneously (Fig. 2). Globally, the microbiota of *M. ulcerans*-PCR positive samples
231 was statistically enriched for Actinobacteria (Fisher's test p-value = 0.03). The genus *Aspergillus* and the
232 species *Aspergillus flavus* were significantly associated with *M. ulcerans*-PCR positive samples (two-
233 sided Fisher's exact test p-value = 0.0021 and 0.015, respectively), and the genus *Penicillium* was
234 significantly associated with *M. ulcerans*-PCR negative samples (p-value = 0.0021, Fisher's exact test).
235 All *Zygomycetes, Acidovorax, Brevundimonas, Cutibacterium*, and *Homoserinibacter* species were
236 recovered from PCR *M. ulcerans*-positive samples, whereas all *Penicillium, Cellulosimicrobium,*
237 *Franconibacter, Ochrobactrum, Porphyromonas, Roseomonas, Achromobacter* and *Lelliottia* species
238 were recovered from *M. ulcerans*-PCR-negative samples, but these associations were not statistically
239 significant (Fig. 2). The dendrogram obtained by the agglomerative hierarchical classification of the
240 species recovered from samples harboured two distinct clusters perfectly separating the *M. ulcerans*-
241 PCR positive samples from the *M. ulcerans*-PCR negative samples (Fig. 3). These results highly suggest
242 that the skin microbiota was significantly correlated with *M. ulcerans* carriage.
243 **Principal component analysis.** After submission of the results of fungal and bacterial
244 culturomics and PCR *M. ulcerans* to PCA analysis, two varifactors (F1 and F7; representing both
245 a total of 28.3% of the variance of the data) were selected because these two varifactors

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246 represented the highest variance of the variable "PCR *M. ulcerans*". F1 and F7 contributed 20.4%
247 and 7.9% of the overall variability, respectively. *Aspergillus flavus*, *Propionimicrobium*
248 *lymphophilum* and *Propionimicrobium freudenreichii* were strongly associated with a positive
249 PCR for *M. ulcerans,* whereas *Penicillium rubens*, *Penicillium chrysogenum* and *Lelliota*
250 *nimipressuralis* were associated with a negative PCR. Surprisingly, detection of *Aspergillus* fungi
251 positively correlated with PCR detection of *M. ulcerans*. Indeed, *A. flavus* was the fungus
252 exhibiting the strongest positive association with *M. ulcerans*. On the other hand, and
253 unexpectedly, we observed that the detection of *Penicillium* fungi was anti-correlated with the
254 detection of *M. ulcerans*. No *Penicillium* detection positively correlated with *M. ulcerans*
255 detection, while *P. rubens* and *P. chrysogenum* were the only fungi to anti-correlate with *M.*
256 *ulcerans* (Fig. 4).

257 **Factor discriminant analysis.** Interestingly, factor discriminant analysis identified five fungal
258 and bacterial species that significantly discriminated *M. ulcerans*-PCR positive and negative
259 samples, including *A. flavus, Brevundimonas diminuta, Propionimicrobium lymphophilum,*
260 *Pantoea dispersa* and *P. rubens* (p-values: 0.001, 0.01, 0,049, 0.049, 0.049, respectively –Table
261 S2). The discriminant analysis showed that the cutaneous fungal and bacterial microbiota
262 discriminated with 100% accuracy between *M. ulcerans* positive and negative groups (receiver
263 operator curve analysis: area under curve = 1 - no error in the confusion matrix – perfect
264 clustering).

265 **Fungi-*M. ulcerans* biological interactions**. Mycolactones significantly increased spore germination of
266 *A. flavus* after a 10-hour incubation (stimulated 91.2% vs. control 28.75%; P <.0001), significantly
267 increased spore germination of *A. niger* (stimulated 75.42% vs. control 69.17%; P <.0001), and
268 significantly decreased spore germination of *P. rubens* (stimulated 45.2% vs. control 50.7%; P <.0001)

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269 (Fig. 5). Moreover, we observed that mycolactones significantly attracted *A. flavus* and *A. niger* during
270 the T-test assay (*P*-value: 0.013 and 0.003, respectively) (Fig. 6).
271

272 DISCUSSION

273 Using a standard RT-PCR assay to detect the asymptomatic skin carriage of *M. ulcerans* in skin samples
274 collected from individuals living in Burkina Faso, we detected a prevalence of 11.5% of *M. ulcerans*
275 carriage in individuals residing in the Sindou region, Burkina Faso, where Buruli ulcer cases have been
276 previously reported [20]. Our data agree with a previous report of cutaneous carriage of *M. ulcerans* in
277 Ghana among people practising agriculture without any protective clothing and in infants crawling on
278 the soil [28]. Thus, the data reported here confirm that in Buruli ulcer-endemic areas, some people are
279 asymptomatic skin carriers of *M. ulcerans*. The *M. ulcerans* inoculum we detected on the skin has been
280 reported to be sufficient to initiate Buruli ulcer lesions in a murine model [29]. These observations
281 suggest that asymptomatic skin carriage could be a previously undescribed condition in the natural
282 history of Buruli ulcer.

283 Exploration of the skin microbiota at the interface of healthy and diseased skin is in its infancy,
284 especially regarding Buruli ulcer [30]. Comparison of skin microbiota between *M. ulcerans*-PCR
285 positive and negative samples revealed a specific cutaneous microbiota associated with asymptomatic *M.*
286 *ulcerans* skin carriage, even predicting *M. ulcerans* skin carriage. Other skin pathogens and diseases
287 (leprosy and psoriasis) have been previously related to a specific skin microbiota [31-32]. The novelty in
288 our study was to find significant, antiparallel associations between *M. ulcerans* and fungi: *M. ulcerans*
289 was detected on the skin along with *Aspergillus*, in which spore germination and the fungal network
290 were stimulated by mycolactones, whereas the detection of *M. ulcerans* has never been associated with
291 the the presence of *Penicillium* species in the skin, Accordingly, spore germination of *P. rubens* was
292 inhibited by mycolactones. Therefore, our clinical observations were not fortuitous but revealed

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293 transkingdom mycobacteria-fungi interactions, supporting preliminary observations made with the
294 Zygomycete *Mucor circinelloides* [26]. Of note, the transkingdom communication concerned *P. rubens,*
295 a penicillin-producing strain, alias *P. chrysogenum*, made famous by Sir Alexandre Fleming [33].

296 All these observations suggest for the first time that transkingdom communications between fungi
297 and mycobacteria are of medical interest, partially driving the natural history of Buruli ulcer. These
298 observations will stimulate additional studies to disclose whether this holds true for other mycobacterial
299 infections of medical interest.
300

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398 **FIGURE LEGENDS**

399 Fig. 1: Real-time PCR detection of *M. ulcerans* from 52 swabs collected in the Sindou region,
400 IS*2404* and IS*2606* are specific insertion sequences for *M. ulcerans* KR-B ketoreductase-B gene.

401 Fig. 2: The different microorganisms grown from 12 skin samples comprising 6 *M. ulcerans*
402 positive samples and 6 negative samples are presented with their relative frequency differences
403 and associated P-values. A total of 84 species distributed among 62 bacteria and 22 fungi were
404 cultured.

405 Fig. 3: The agglomerative hierarchical classification dendrogram of individuals according to their
406 cutaneous microbiota associated with the detection (P1 to P6) or absence (N1 to N6) of *M.*
407 *ulcerans. M. ulcerans* PCR-negative samples and *M. ulcerans* PCR-positive samples were clearly
408 separated into two distinct clusters according to the composition of the skin microbiota.

409 Fig. 4: PCA of 84 bacterial and fungal species isolated from individuals in Burkina Faso revealed
410 a strong association of *A. flavus*, *P. lymphophilum* and *P. freudenreichii* with *M. ulcerans* skin
411 carriage.

412 Fig. 5: Mycolactone-induced spore germination of *A. flavus*, *A niger* and *P. rubens* in the
413 presence of PBS as a negative control.

414 Fig. 6: Fungi attraction test: mycolactones (right side disk) attract *A. flavus* and *A. niger* in the
415 presence of PBS as a negative control (left side disk).

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416 **Supplementary data**

417 Supplementary Table 1: Primers used in this study for the PCR-based identification of fungi and

418 bacteria.

	Name	Sequences 5'-3'
Fungal sequences	ITS3	GCATCGATGAAGAACGCAGC
	ITS4	TCCTCCGCTTATTGATATGC
	Bt2a	GGTAACCAAATCGGTGCTGCTTTC
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC
	Al33F	GAYTTCATCAAGAACATGAT
	Al33R	GACGTTGAADCCRACRTTGTC
	Al34F	TTCATCAAGAACATGAT
	Al34R	GCTATCATCACAATGGACGTTCTTGGAG
	ITS1	TCCGTAGGTGAACCTGCGG
	ITS2	GCTGCGTTCTTCATCGATGC
Bacterial sequences	FD1	AGAGTTTGATCCTGGCTCAG
	rP2	ACGGCTACCTTGTTACGACTT
	536F	CAGCAGCCGCGGTAATAC
	536R	GTATTACCGCGGCTGCTG
	800F	ATTAGATACCCTGGTAG
	800R	CTACCAGGGTATCTAAT
	1050F	TGTCGTCAGCTCGTG
	1050R	CACGAGCTGACGACA

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421 Supplementary Table 2: Factor discriminant analysis (FDA) revealed five fungal and bacterial
422 species that significantly discriminated *M. ulcerans*-PCR positive and negative samples.
423

Variable	Lambda	F	DDL1	DDL2	p-value
Propionimicrobium lymphophilum-0	0,667	5,000	1	10	0,049
Brevundimonas diminuta-0	0,500	10,000	1	10	0,010
Pantoea dispersa-0	0,667	5,000	1	10	0,049
Penicillium rubens-0	0,667	5,000	1	10	0,049
Aspergillus flavus-0	0,286	25,000	1	10	0,001

424
425

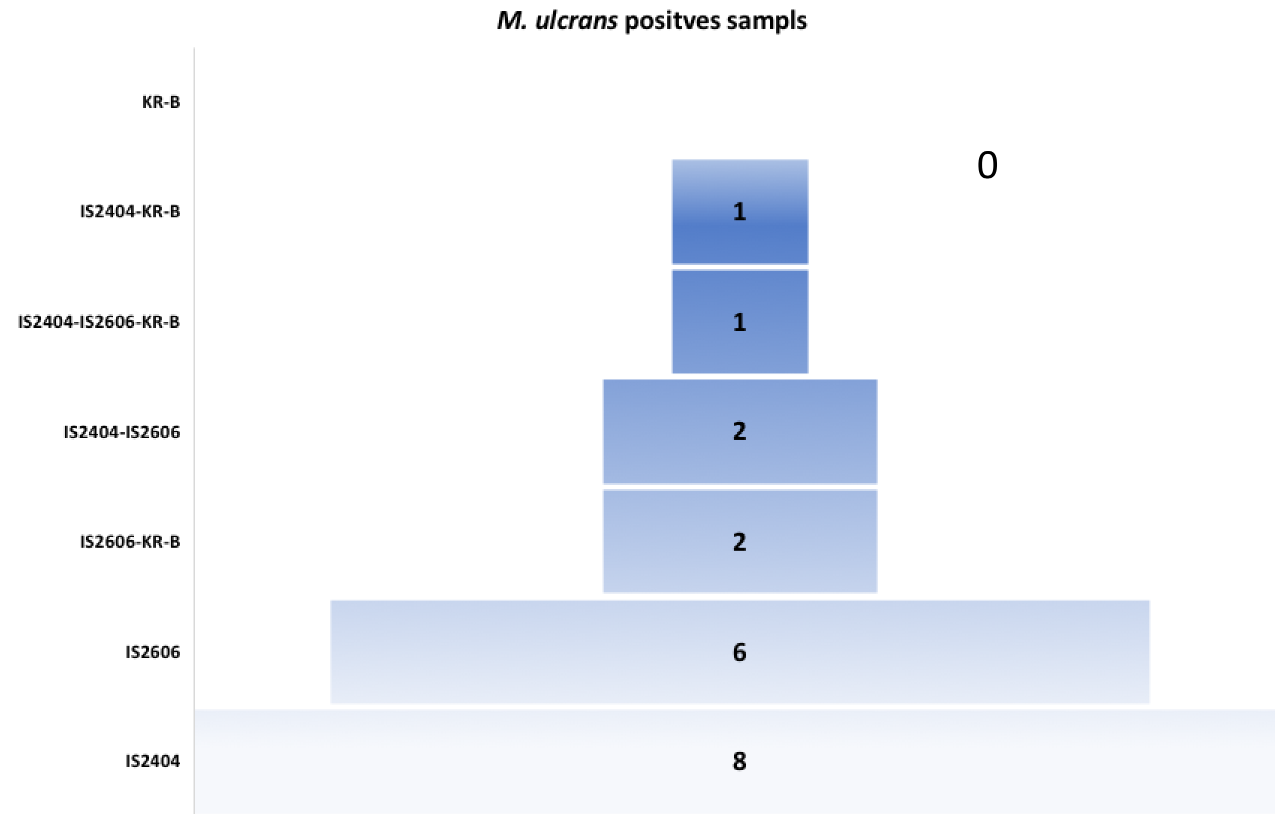


Fig. 1.

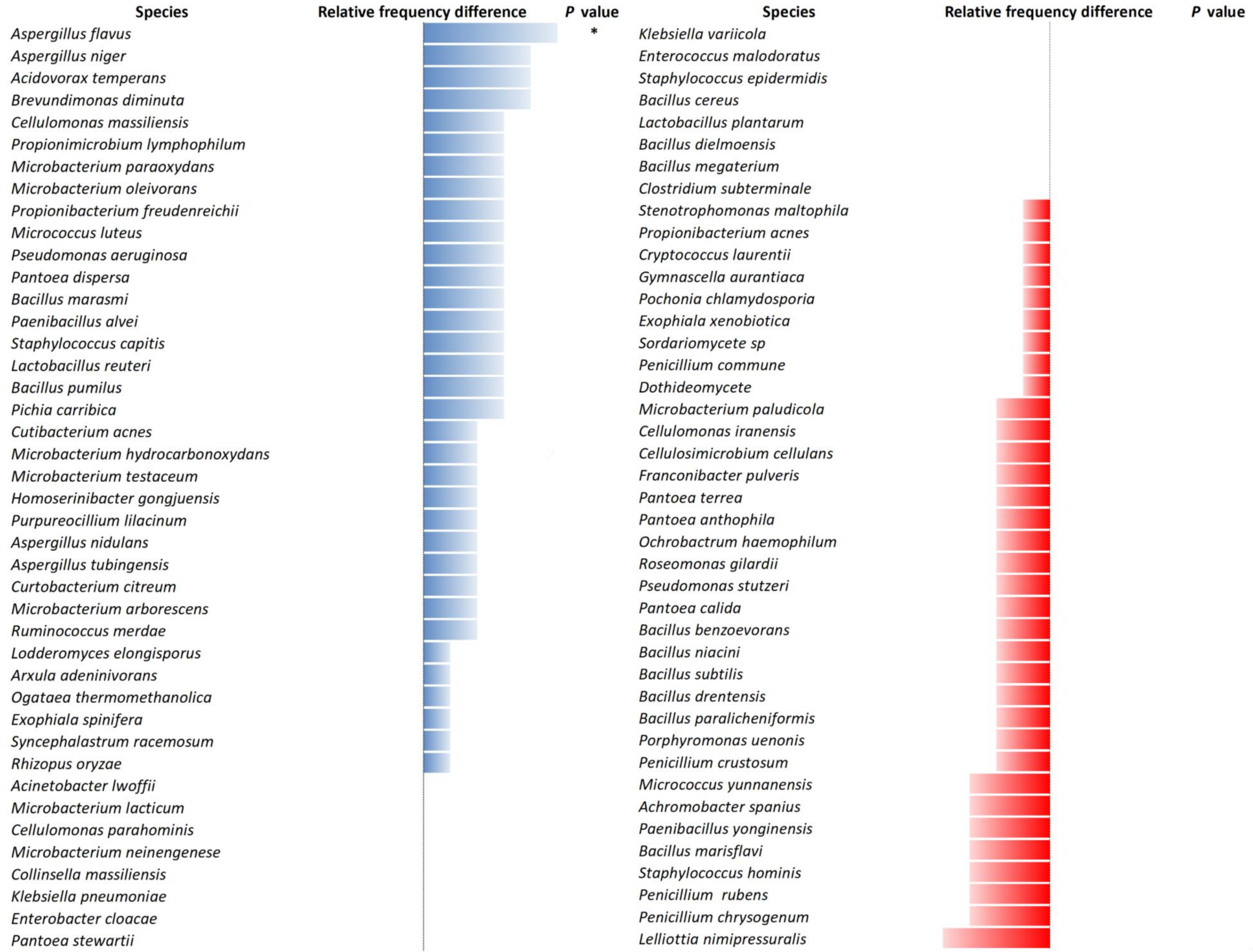


Fig. 2.

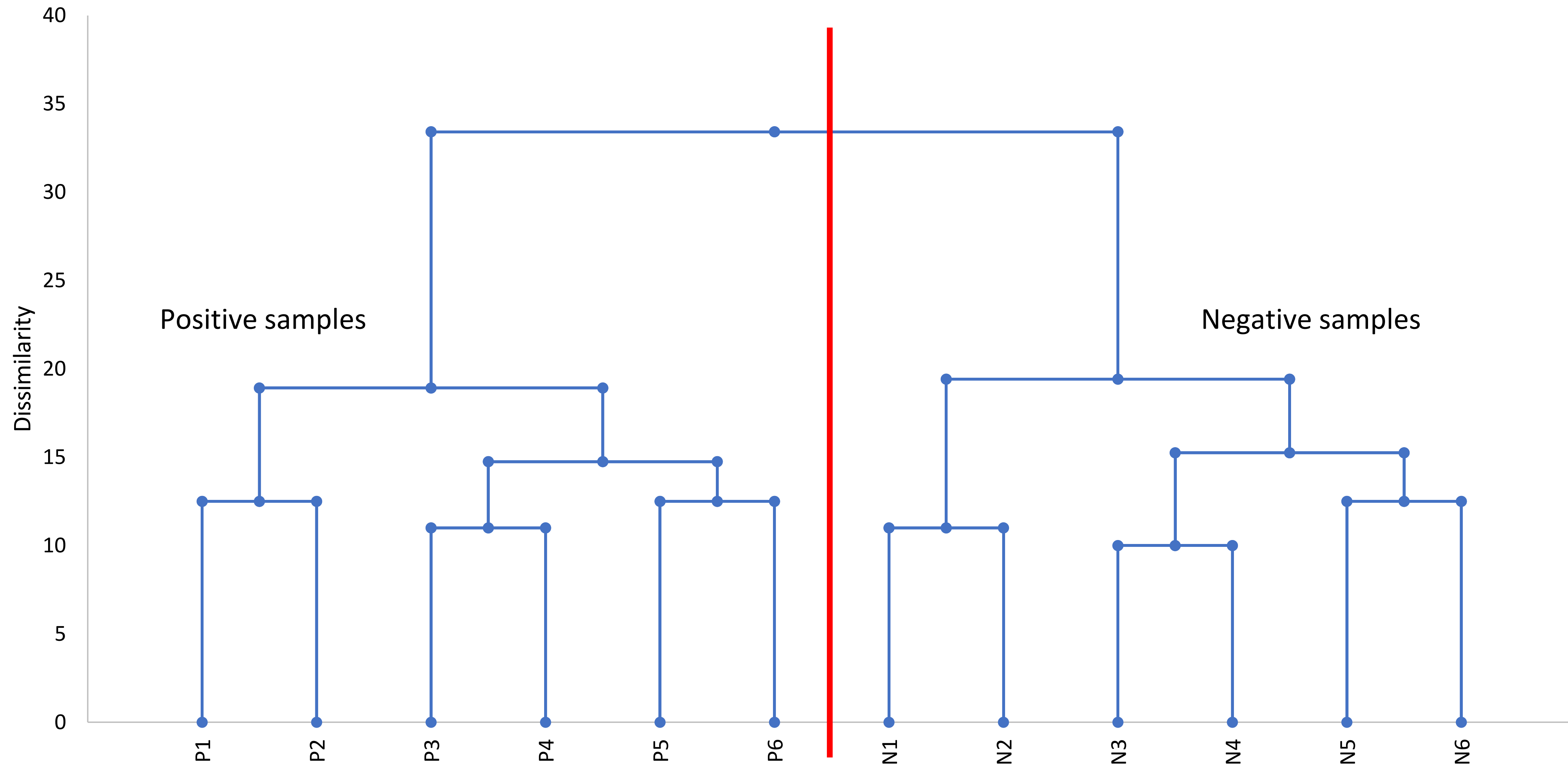


Fig. 3.

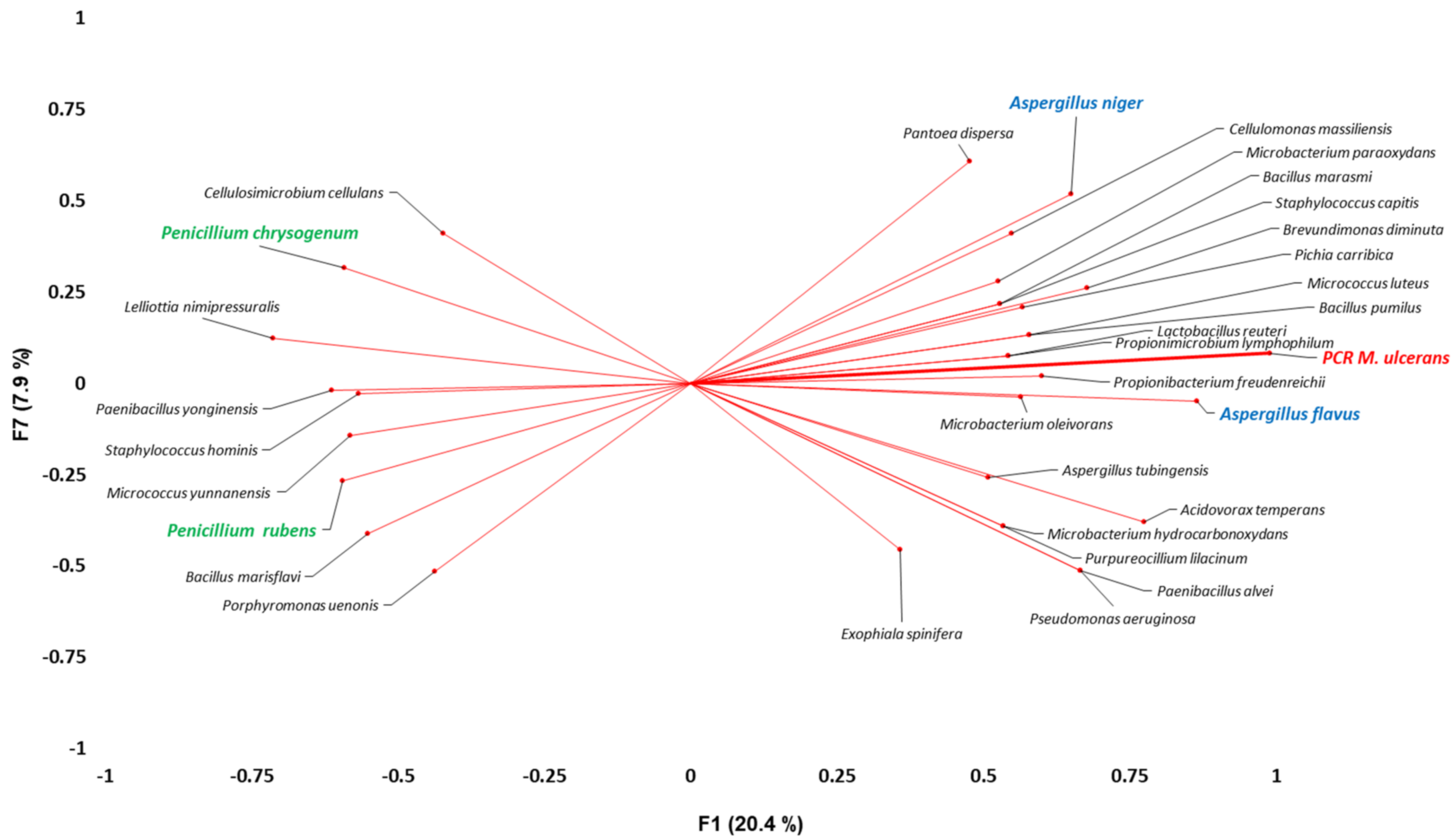
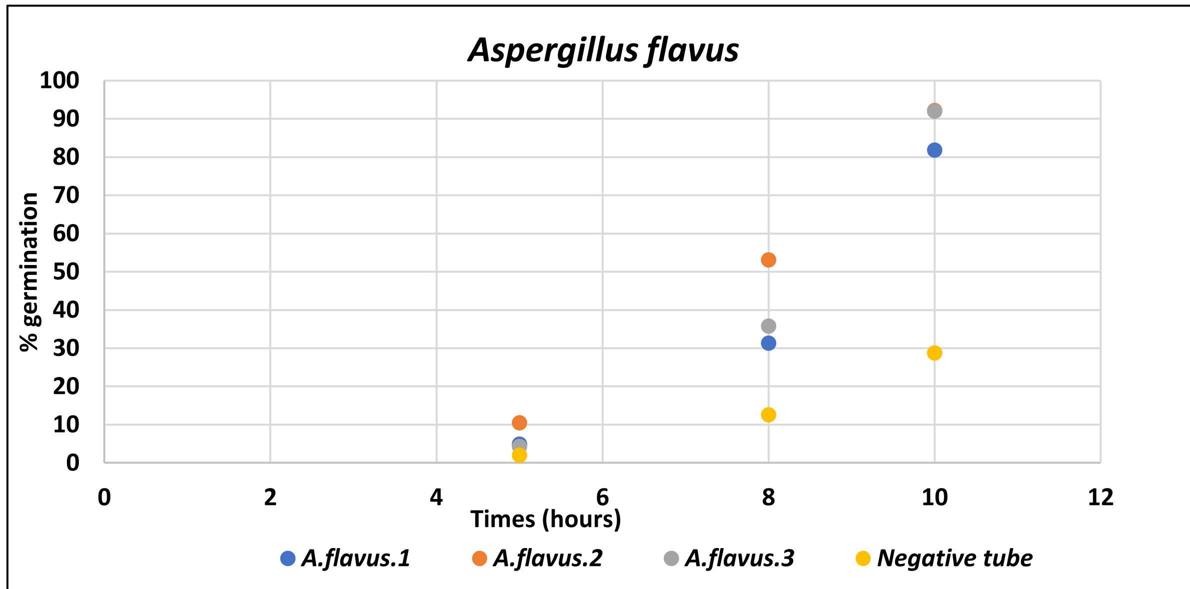
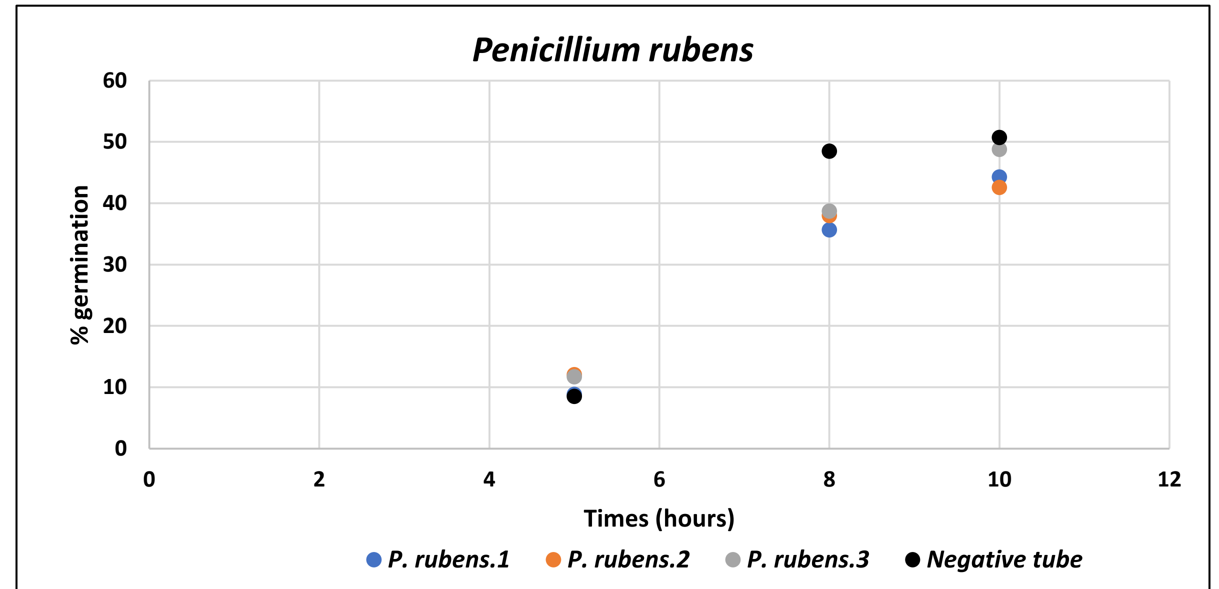


Fig. 4.





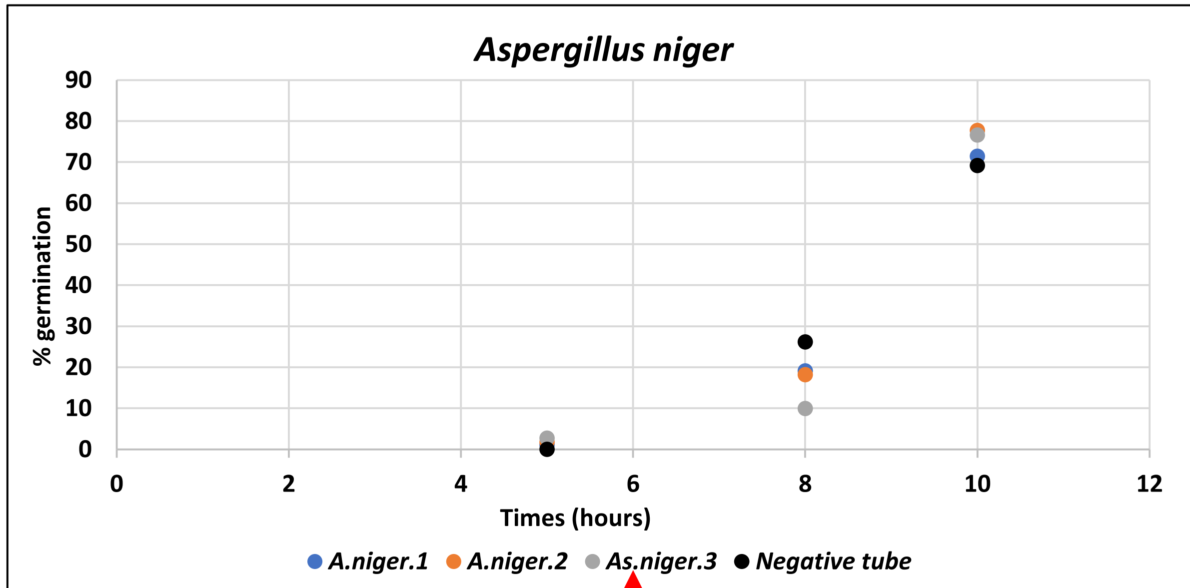
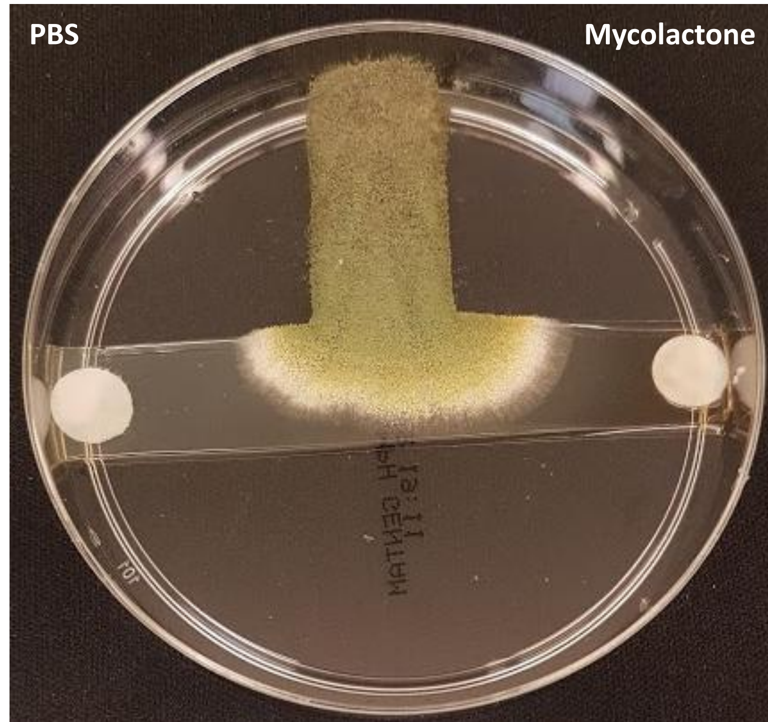


Fig. 5.



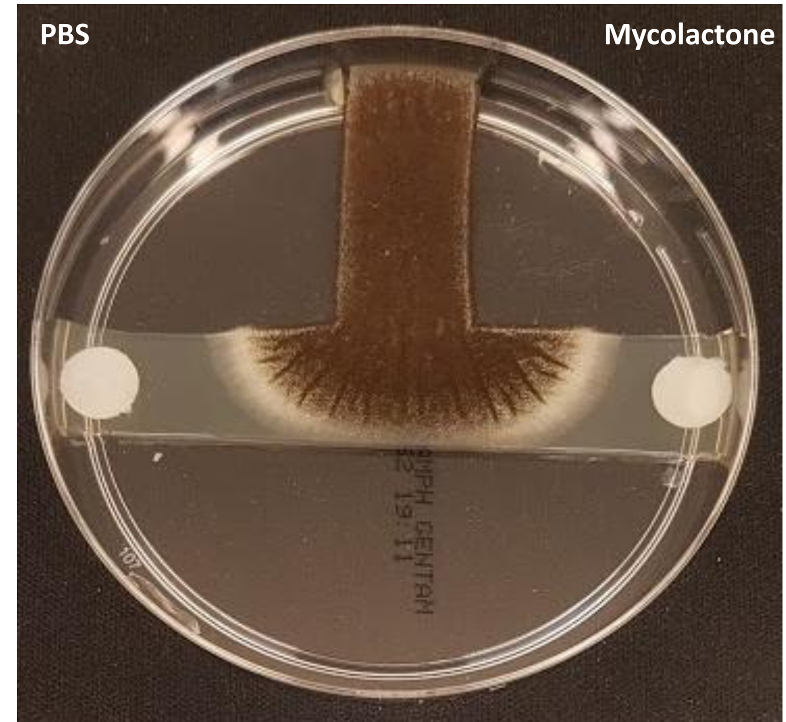


Fig. 6.