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Bacterial biofilms on thalli of Laboulbeniales: a community uncovered

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Bacterial biofilms have been described on a number of fungal taxa. These microbial communities are of interest both from an ecological and a biotechnological point of view, as they have been shown to play a role in biodegradation and biosynthesis. This study is the first to show the presence of biofilms on thallus surfaces of Laboulbeniales, an order of fungi that have biotrophic associations with arthropod hosts. Scanning electron microscopy micrographs show an abundance of bacterial biofilms on thalli of three species: *Laboulbenia collae* associated with *Paranchus albipes* (Carabidae), *L. flagellata* associated with *Limodromus assimilis* (Carabidae), and *Hesperomyces virescens* s.l. associated with *Harmonia axyridis* (Coccinellidae). These bacterial communities were mainly found on the thalli, and only in small quantities on the arthropod integument. We suggest genetics and metabolomics approaches to investigate possible interactions between Laboulbeniales fungi and the biofilms. Our work has laid a foundation for future research on biofilms on Laboulbeniomycetes.

Keywords: Bacterial–fungal interactions, ectoparasitic fungi, scanning electron microscopy, symbiosis.

Biofilms can be found on a plethora of biotic surfaces, such as epithelial cells and fungal filaments (Gaddy & Actis 2009), and abiotic surfaces, such glass and stainless steel (Marques et al. 2007). These multicellular communities are secured by self-produced extracellular biopolymers, providing structure and protection (Seneviratne et al. 2008). These interactions are what make biofilms physiologically distinct from planktonic cells of the same species (Costerton et al. 1995). Biofilms have been investigated extensively from an ecological and a biotechnological point of view, as they have been shown to play a role in biodegradation and biosynthesis (Dobbins et al. 1992, Oppermann-Sanio & Steinbüchel 2002). Biofilm production is not only restricted to bacterial taxa; it has also been observed in green algae with cyanobacteria (García-Meza et al. 2005), fungi (Reichhardt et al. 2016), and other groups of organisms (van Wolferen et al. 2018). Furthermore, biofilms often comprise multiple species interacting with both each other and their surroundings (Davey & O'toole 2000).

Bacterial–fungal interactions have been studied in a large number of taxa (Deveau et al. 2018). For instance, soil fungi were found to have various bacterial associates (Warmink & van Elsas 2009). These communities can consist of up to several hundreds of bacterial species (Deveau et al. 2018). Bacterial communities also play an important role in the lichen symbiosis, fulfilling essential functions such as nutrient supply, resistance against biotic stress factors, and detoxification of metabolites (Grube et al. 2015). Filamentous fungi can harbor distinguished sets of microbiomes for various differentiated tissues, such as ascomata, basidiomata, and mycorrhizae (Zagriadskaia et al. 2013, Deveau et al. 2016, El-Jurdi & Ghannoum 2017). Bacterial microbiota associated with fungi have also been found to contribute to the biology of the host. For instance, when fungi were treated with antibiotics suppressing or altering bacterial communities, various processes, such as mycelial growth and secondary metabolite production, were impaired (Vahdatzadeh et al. 2015, Schulz-Bohm et al. 2017).

Laboulbeniales (Ascomycota: Laboulbeniomycetes) are characterized by a biotrophic lifestyle on arthropods, determinate growth, lack of an asexual stage, high species richness, and intractability to culture (Haelewaters et al. 2015, 2021b). One major difference compared with other members of Ascomycota is that these fungi do not form hyphae. Instead, they form thalli, 3-dimensional, multicellular units of 1000s of cells that can be observed via standard light microscopy approaches (Blackwell et al. 2020). Laboulbeniales are associated with a broad spectrum of species within three arthropod subphyla: Chelicerata, Myriapoda, and Hexapoda (Haelewaters et al. 2021a).

Light microscopy is the most popular technique for morphological and developmental studies of Laboulbeniales. Other techniques that thus far have been used to visualize structures of Laboulbeniales are scanning and transmission electron microscopy (SEM, TEM) (Weir & Beakes 1996, Garcés et Williams 2004, Harwood et al. 2006, Santamaría et al. 2014, Tragust et al. 2016, Reboleira et al. 2018) and X-ray microtomography (Perreau et al. 2021). Tragust et al. (2016) investigated the mode of attachment and possible penetrating structures of Laboulbeniales associated with ants. SEM has been used to identify *Hesperomyces virescens* based on morphological characters (Garcés & Williams 2004, Harwood et al. 2006) and study its developmental stages (Weir & Beakes 1996). Finally, rotational scanning electron (rSEM) micrographs were used for a more advanced visualization of *Thaxterimyces baliensis* on its millipede host (Reboleria et al. 2018). Thus far, possible bacterial–fungal interactions have never been studied in Laboulbeniales

Material and methods

Infected ground beetles (Coleoptera: Carabidae), and ladybirds (Coleoptera: Coccinellidae) were observed using SEM (Tab. 1). Firstly, fresh carabids were collected on marshes of the Scheldt river in a wooded freshwater tidal area in Hingene (Bornem, Belgium) on 8 June 2021. The specimens were sent alive to the Institute of Biology at Leiden University on 10 June and killed using ethyl acetate vapor (Herome Cosmetics, Almere, The Netherlands) on 14 June. Fresh specimens of *Harmonia axyridis* infected by *Hesperomyces virescens* s.l. were collected on *Philadelphus coronarius* plant hosts (Cornales: Hydrangeaceae) in České Budějovice (Czech Republic) on 8 June 2021, sent on 14 June, and killed on 24 June. Secondly, specimens of *Limodromus assimilis* infected by *Laboulbenia flagellata* and of *Halyzia sedecimguttata* infected by *Hesperomyces halyziae* were stored in 70–99 % ethanol for a prolonged period of time before the next step.

First, all specimens were placed for 1 h in a fixation solution (2 % v/v glutaraldehyde 2 % v/v formaldehyde in 1M sodium cacodylate). Next, the host specimens stored in ethanol were transferred from fixation to a critical point dryer specimen holder and dehydrated using 90 % acetone (Honeywell, Muskegon, MI, USA). After incubation for 10 min at room temperature, the critical point dryer specimen holder was placed in 100 % acetone for 1 h. The medium was refreshed twice to remove all remaining ethanol. Freshly collected host specimens were transferred from fixation to 70 % acetone for 1 h. After refreshing, the specimens were stored overnight in 70 % acetone. The specimens were then placed in a critical point dryer specimen holder and washed in three different acetone concentrations (3×20 min in 80 % acetone, 3×20 min in 90 % acetone, 3×20 min in 100 % acetone). After incubating for 10 min at room temperature, the critical point dryer specimen holder was placed in 100 % acetone for 1 h. The metal column was then dried with carbon dioxide in a Bal-Tec CPD 030 critical point dryer (Bal-Tec, Delft, The Netherlands).

Each specimen was attached with double-sided tape to a metal holder. Specimens were subsequent-

Tab. 1. Specimens examined during this study using scanning electron microscopy.

Specimen label	Host species	Laboulbeniales species	Transport to lab
D. Haelew. 3033	*Halyzia sedecimguttata*	*Hesperomyces halyziae*	Ethanol
D. Haelew. 3654	*Harmonia axyridis*	*Hesperomyces virescens* s.l.	Fresh
D. Haelew. 3655	*Harmonia axyridis*	*Hesperomyces virescens* s.l.	Fresh
D. Haelew. 3322	*Limodromus assimilis*	*Laboulbenia flagellata*	Ethanol
D. Haelew. 3657	*Limodromus assimilis*	*Laboulbenia flagellata*	Fresh
D. Haelew. 3664	*Paranchus albipes*	*Laboulbenia collae*	Fresh

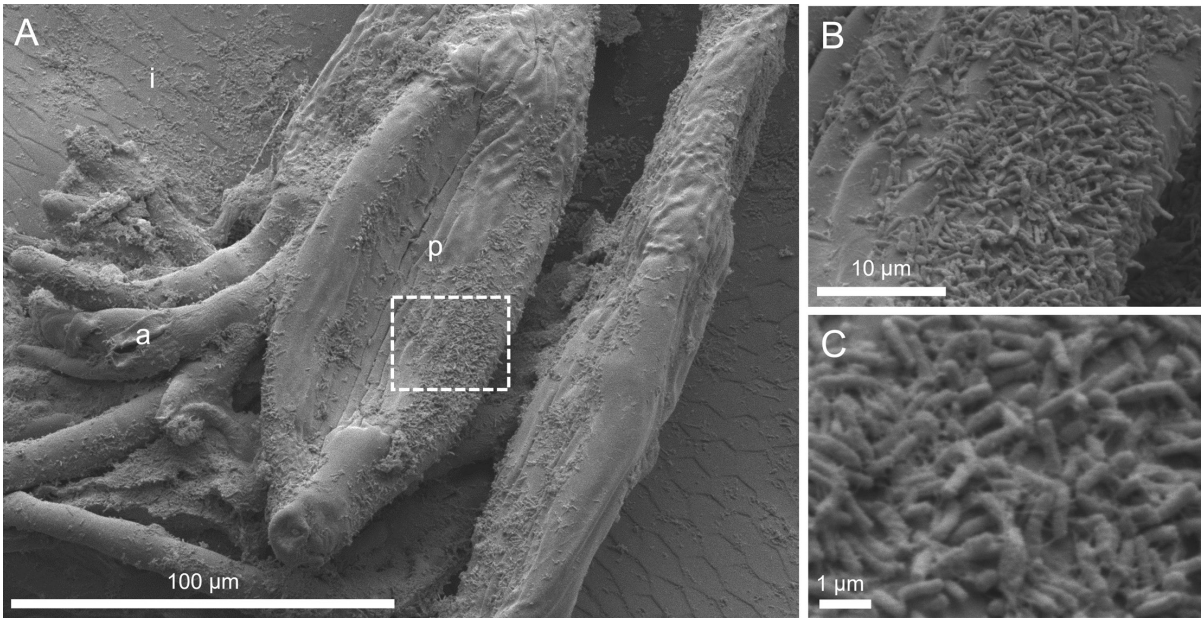

Fig. 1. Bacterial biofilms on *Laboulbenia flagellata* infecting *Limodromus assimilis* (D. Haelew. 3657). **A.** Two mature thalli. **B.** Perithecium wall covered with bacteria. **C.** Close-up of biofilm present on the perithecium. Abbreviations: a appendages, i integument, p perithecium.

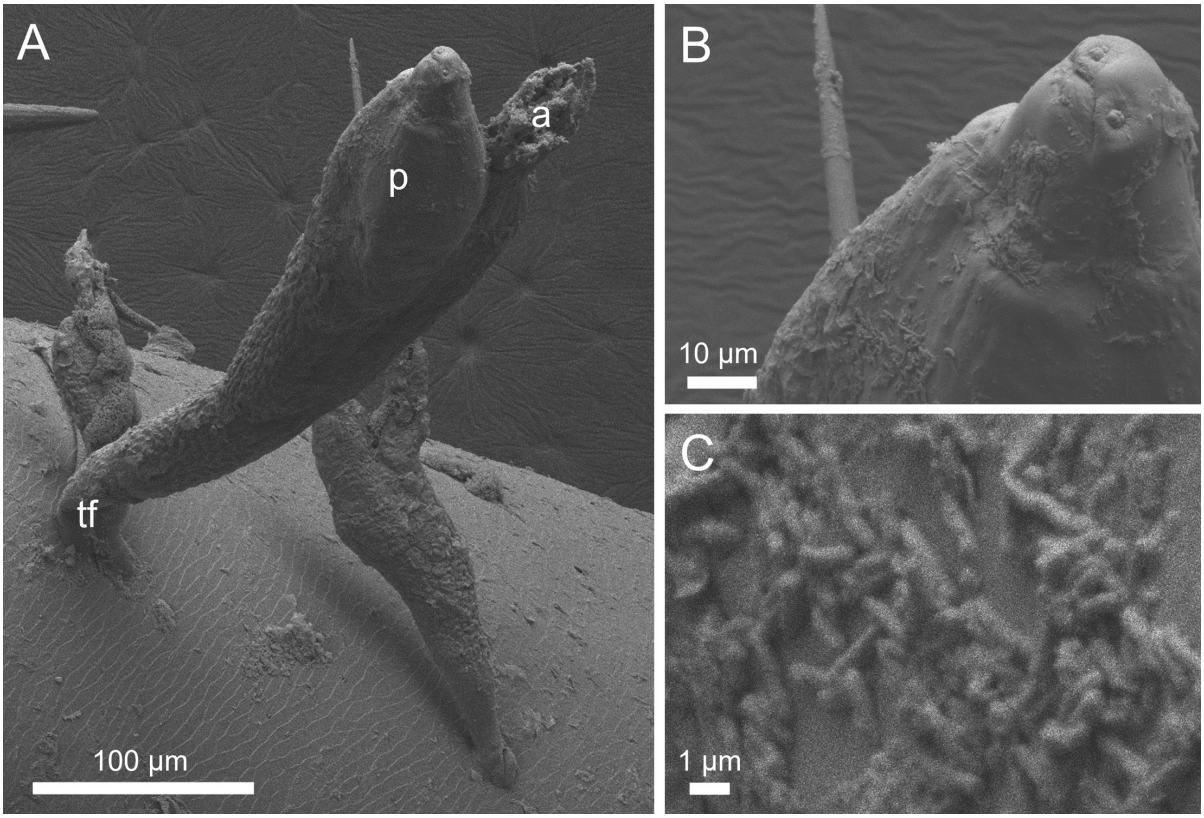

Fig. 2. Bacterial biofilms on *Laboulbenia collae* infecting *Paranchus albipes* (D. Haelew. 3664). **A.** Two mature thalli. **B.** Perithecium covered with bacteria. **C.** Close-up of biofilm present on the perithecium. Abbreviations: a appendages, p perithecium, tf thallus foot.

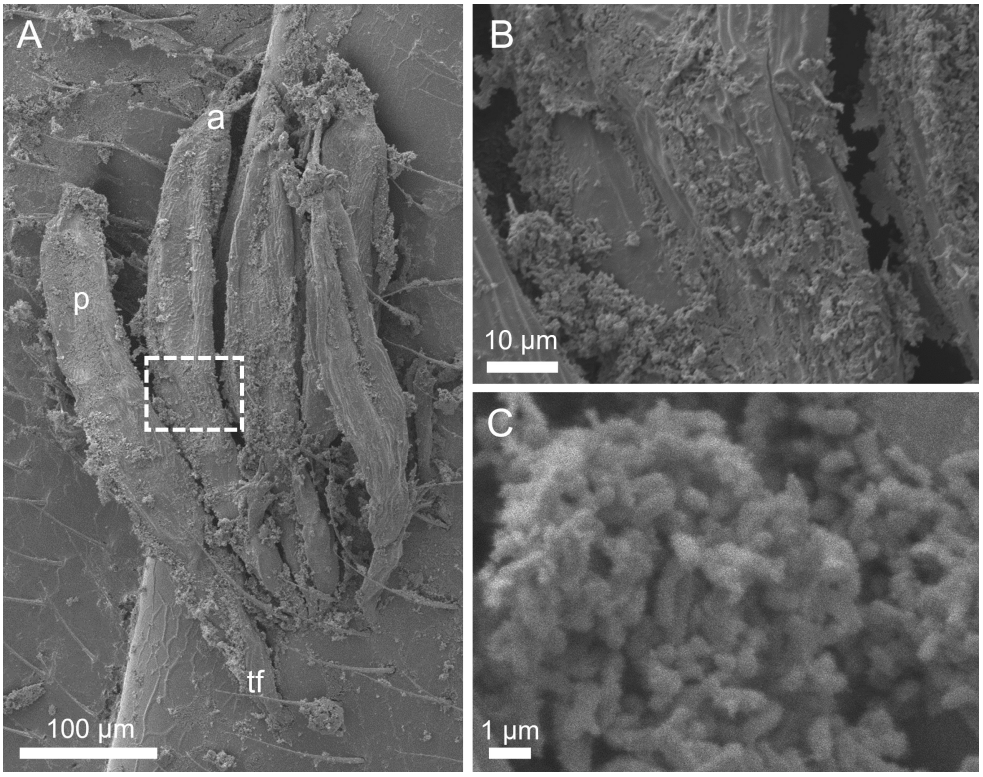

Fig. 3. Bacterial biofilms on *Hesperomyces virescens* infecting *Harmonia axyridis* (D. Haelew. 3654). **A.** Five mature thalli. **B.** Perithecium covered with bacteria. **C.** Close-up of biofilm. Abbreviations: a appendages, p perithecium, tf thallus foot.

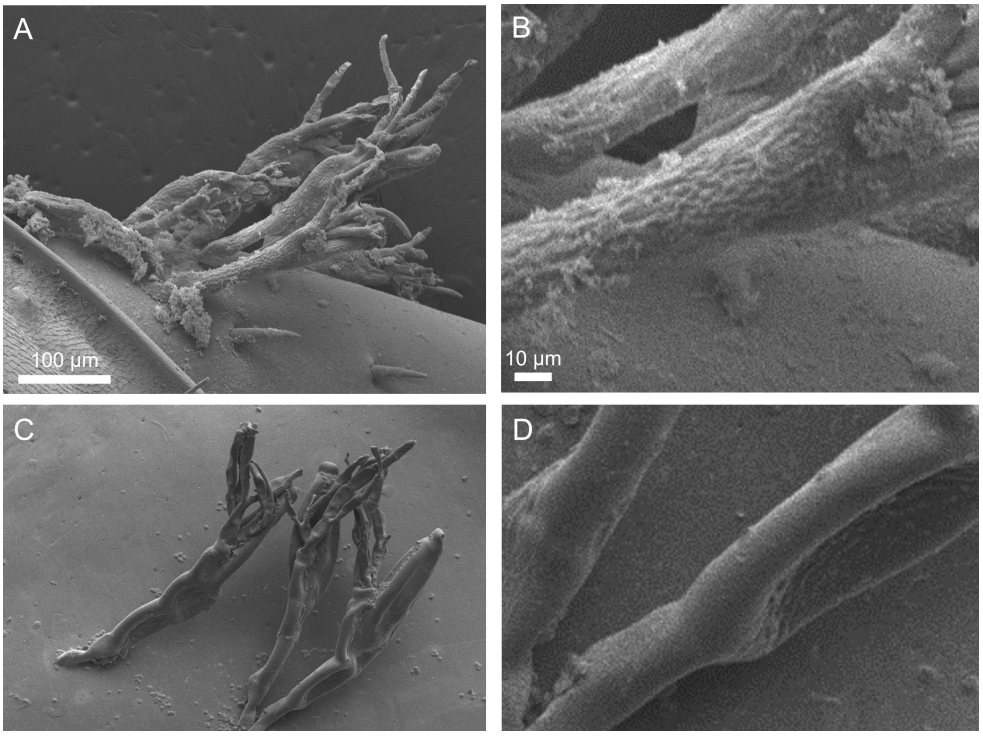

Fig. 4. *Laboulbenia flagellata* infecting *Limodromus assimilis*. **A–B.** Thalli on a freshly killed and fixed host specimen (D. Haelew. 3657). **C–D.** Thalli on a host (D. Haelew. 3322) stored in ethanol.

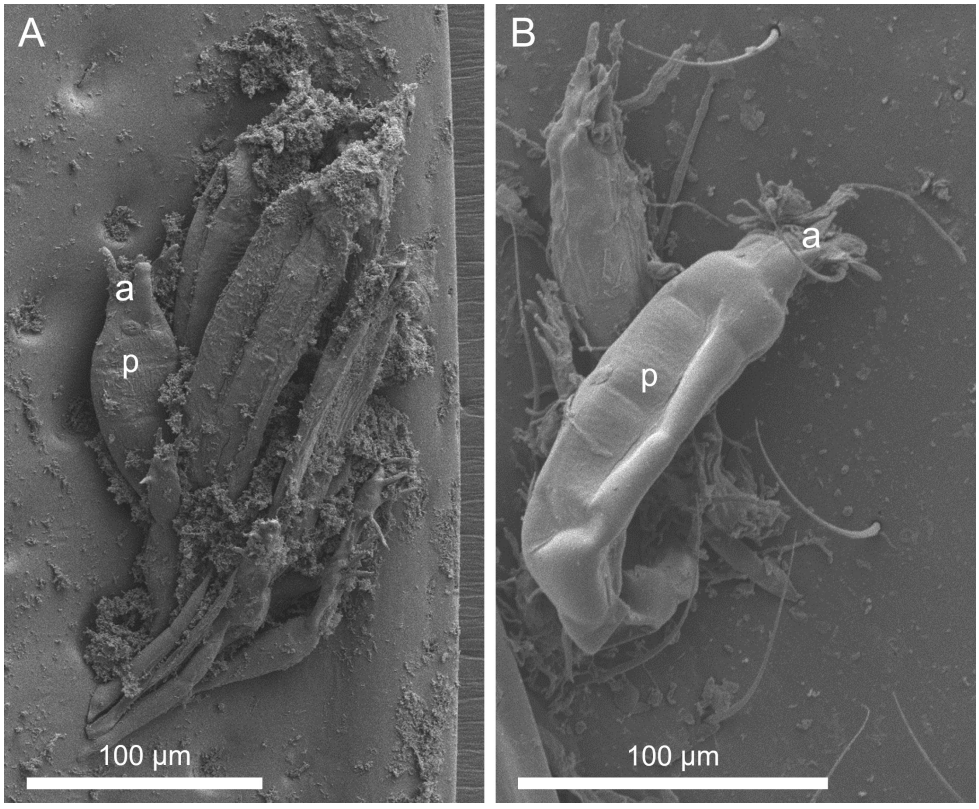

Fig. 5. *Hesperomyces* spp. infecting ladybirds. **A.** Thalli of *H. virescens* s.l. on freshly killed and fixed *Harmonia axyridis* (D. Haelew. 3655). **B.** Thalli of *H. halyziae* on *Halyzia sedecimguttata* stored in ethanol (D. Haelew. 3033). Abbreviations: a appendages, p perithecium.

ly loaded in a Q150T Plus turbomolecular pumped coater (Quorum Technologies Ltd, East Sussex, UK) and coated with a layer of platinum–palladium of 20-nm thickness. Finally, the specimens were observed in a JSM-7600F Schottky field emission scanning electron microscope (JEOL, Zaventem, Belgium) at 5.0 kV.

Results

SEM micrographs revealed the presence of bacterial biofilms on thallus surfaces of Laboulbeniales. All observed bacteria had a rod-shaped morphology with an average length of 2 µm. Biofilms were clearly present on the thalli, but barely on the insect integument itself (Figs. 1–3, 4A–B, 5A). The biofilms were only observed on the surface of thalli attached to insects that had been collected alive, killed, and then quickly stored in fixation chemicals. Biofilms were observed on all parts of the thallus, although most abundant on the perithecium. On specimens that had been stored in ethanol, biofilms were absent (Figs. 4C–D, 5B).

Discussion

This study is the first to show the presence of biofilms on Laboulbeniales. Richards & Smith (1955) did mention gram-negative, bipolar-staining, short-rod bacteria in the interior of mature perithecia of *Herpomyces stylopygae* (Herpomycetales). One reason that biofilms have not been reported previously may be that these rod-shaped bacteria are too small to observe with a compound microscope, traditionally the most common microscope in use for Laboulbeniales research. However, biofilms have also not been observed in previous electron microscopy research (Weir & Beakes 1996, Harwood et al. 2006, Garcés & Williams 2004, Tragust et al. 2016, Santamaría et al. 2014, Reboleira et al. 2018). Biofilms, if they were present, were most likely eradicated because the infected insects were stored in ethanol. Previous research has shown the effectiveness of ethanol on removing biofilms (Peters et al. 2013). This was also the case for our initial scans, which were taken of specimens that had been preserved in ethanol for longer than a year. It was ap-

parent that the surface was completely clean aside from organic, non-living debris (Fig. 4C–D). On the specimens that were freshly collected, killed, and quickly stored in fixation chemicals however, the biofilms could be fixed during the procedure without losing structure.

Therefore, for future experiments on bacterial associations with insect-associated fungi, we think it better to directly fix freshly-killed infected arthropod specimens in fixation chemicals. They should not be stored in ethanol for the following reasons. First, it takes a significantly longer time to dry the specimens as all ethanol must be replaced by acetone prior to critical point drying. Otherwise, drift might occur, meaning that micrographs at a certain magnification are distorted by the motion of the sample during image acquisition (Jin & Li 2015). Second, ethanol can extract water and fat from structures that in fresh specimens can be clearly seen (Figs. 4C–D, 5B). We assume these 'imploded' structures are especially present in specimens stored for prolonged time in ethanol. Third, as mentioned before, bacterial biofilms can be washed by ethanol through denaturation of proteins (Peters et al. 2013).

Bacterial communities were predominantly found on thallus surfaces, and only few bacteria were found on the arthropod integument. The bacteria could potentially thrive on the thallus surface benefiting from enhanced dispersal and growth on fungal exudates (Warmink & Van Elsas 2009, Lohberger et al. 2019). However, Laboulbeniales thalli might also consume bacteria as has also been suggested for, e.g., *Agaricus bisporus* and slime molds (Castillo et al. 2011, Vos et al. 2017, Kertesz & Thai 2018). It would therefore be of interest to investigate (*i*) whether these biofilms consist of one or multiple species; (*ii*) whether the species composition of the biofilms may be dependent on the fungus, arthropod host, or habitat; and (*iii*) whether these bacteria represent mutualistic or pathogenic species. Bacterial inoculation on Lysogeny broth (LB) agar and Sanger sequencing or high-throughput sequencing approaches focusing on the 16S of the ribosomal RNA gene will be necessary to identify the species that form these biofilms (e.g., Janda & Abbott 2007, Chamberland et al. 2017). Besides, these communities could produce certain antimicrobial molecules to inhibit growth of other bacterial and fungal species; metabolomics approaches could be employed to investigate possible bioactive compounds produced by the bacteria.

Bacteria could perhaps even contribute to the development of ascospores into mature thalli. Culturing attempts of Laboulbeniales have thus far been proven unsuccessful (reviewed in Haelewaters et al. 2021a). Whisler (1968) was perhaps most successful; he attempted growth on brain-heart-agar medium but thalli of *Stigmatomyces ceratophorus* only developed to a 20-cell stage, producing spermatia but not perithecia. For these culturing experiments, perithecia were washed in several antibiotic rinses after which the ascospores were squeezed out (Whisler 1968). These rinses prevented bacterial growth on the culture media but, conversely, may have contributed to the unsuccessful growth. The presence of a biofilm on the mature perithecium (Figs. 1–2, 5A), especially around the tip, seems a convenient way to inoculate exiting ascospores with bacteria—transferring bacteria from one generation to the next. Co-culturing approaches with bacteria have been shown necessary for growth and fructification of cultivated mushrooms (Garbaye et al. 1992; Noble et al. 2003, 2009). The alternative possibility, the biofilm bacteria being dependent on the fungus, is less probable because the biofilms appear easily on young, developing thalli from the environment and thus they might represent common species and be separately culturable. Ectomycorrhizal fungi also have been described to modify and select the microbiome that is associated with their environmental niche (Li et al. 2017, Liu et al. 2021). For Laboulbeniales, the bacteria could perhaps directly or indirectly provide nutrients necessary for growth into mature thalli, a hypothesis that, we think, merits further investigation. This can be more important for those Laboulbeniales lacking a haustorium, for mining nutrients from the host's integument (Tragust et al. 2016, Haelewaters & De Kesel 2020, Haelewaters et al. 2022).

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