University of South Bohemia in České Budějovice
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Antifungal potential of volatile compounds of Actinobacteria against a selection of fungal phytopathogens

Bachelor thesis

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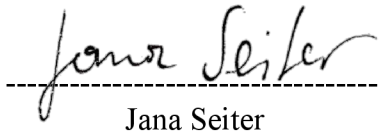
Annotation:

In the present study, the antifungal potential of volatile compounds, produced by a selection of Actinobacteria, was tested against several fungal plant pathogens, belonging to the genus of *Fusarium* and *Geotrichum*. Therefore in vitro experiments were performed, in which two-compartment Petri dishes were used. This was followed by an evaluation of the potential suitability of the volatile producing bacteria for their possible application in biological control of the tested pathogens.

Declaration:

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 3.8.2022



Jana Seiter

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Abstract:

Bacteria inhabiting soil are of great diversity and essential for the health of soil and plants. Among these, Actinobacteria are known to produce a broad range of bioactive metabolites, some of those metabolites are volatile in ambient conditions and therefore they can be important in long distance interactions between microorganisms. Certain volatile metabolites exhibit antimicrobial activity against fungal phytopathogens, which possibly qualifies them for their use in the biological control of fungal plant pathogens. When applied in biological control, their antifungal volatiles could serve as a promising alternative to conventional fungicides. Here, we aimed to test the antifungal potential of the volatile blends produced by selected Actinobacteria against different fungal phytopathogens including *Fusarium* and *Geotrichum* species. To assess the effect of the volatile blends produced by bacteria on the growth of the fungal pathogens, we used in-vitro systems based on two-compartment Petri dishes. This study shows that volatiles of *Streptomyces* ssp. cause a growth reduction of 7-20% among three out of four pathogens, with *Streptomyces coelicolor* A3(2) showing the highest inhibitory capacity (20%). However, we did not find any effect on the pathogens caused by *Kutzneria* sp. 1627. Interestingly, the growth of *Fusarium oxysporum* 0146 was not inhibited by volatiles of any bacterium but promoted by the volatiles of *Streptomyces antibioticus* 2187. Our findings confirm the antifungal activity of volatiles produced by *Streptomyces* species and suggest their possible application in biological control of these pathogens. The model organism *Streptomyces coelicolor* A3(2) could be the future biocontrol agent most promising in this study and the first to inhibit *Geotrichum candidum* growth through bacterial volatiles. Further this study gives insight into the antifungal potential of volatile compounds produced by *Streptomyces* species originating from the digestive tract of millipedes.

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1. Introduction

1.1. Fungal phytopathogens endanger food security

According to projections from the Food and Agriculture Organization of the United Nations (FAO), the world population is expected to grow to nearly 10 billion by 2050, resulting in a higher need for food and resources (FAO 2018; Zhang et al. 2020). Worldwide food security is at risk due to major yield losses caused by fungal plant pathogens (Leannec-Rialland et al. 2022; Zhang et al. 2020). These pathogens include representatives of the genus of *Fusarium*, which is especially harmful when it comes to yield and food security. Some of *Fusarium* species cause a threat to food production by producing mycotoxins, compounds originating from the secondary metabolism of fungi which are harmful to animals and humans (Leannec-Rialland et al., 2022; Lee et al., 2017). Another fungal species responsible for yield losses is *Geotrichum candidum*, which infects different fruits and vegetables and is the causal agent of the postharvest disease sour rot in citrus fruits (Talibi et al., 2012a, 2012b). *Geotrichum* is considered a storage pathogen while *Fusarium* causes both pre- and post-harvest losses (Abu Bakar et al. 2013; Talibi et al. 2012b; Thornton et al. 2010; Tiwari et al. 2021).

1.2. Biological control as an alternative to pesticides

Nowadays, mostly synthetic fungicides are used to combat fungal plant pathogens in agriculture, but their impact on human health and the environment is considered hazardous (Elnahal et al., 2022; Leannec-Rialland et al., 2022; Tudi et al., 2021). In addition, the tighter regulations and restrictions on the application of pesticides, which are initiated by the European Union for example, create the need for more secure and environmentally friendly alternatives to chemical pesticides and thus also fungicides (Lammers et al., 2022; Thambugala et al., 2020). One of these alternative methods is the application of biological control agents, these are organisms which protect crops against pests, weeds and pathogens (Alizadeh et al., 2020; Elnahal et al., 2022; Poveda, 2021; Thambugala et al., 2020; Trivedi et al., 2021). Biological control agents can be microbes, which are suppressive towards phytopathogens in exerting antagonistic mechanisms, and might have beneficial effects on their host plants in supporting their growth and defensive reactions (Alizadeh et al., 2020; Elnahal et al., 2022; Feichtmayer et al., 2017; Köhl et al., 2019; Poveda et al., 2020; Thambugala et al., 2020). As biocontrol agents, native or non-native beneficial microbes can be used (Elnahal et al., 2022). Typically, these include fungi or bacteria that live within their host plants (endophytic), on their surface, or in the soil surrounding their roots (rhizosphere)

(Djebaili, Pellegrini, Bernardi et al., 2021; Elnahal et al., 2022; Sahu et al., 2019; Thambugala et al.; 2020, Tyc et al., 2017).

1.3. Antagonistic interactions in biocontrol

Microbial biocontrol agents, which mostly include bacteria and fungi, can act as pathogen antagonists (Elnahal et al., 2022; Thambugala et al., 2020). Antagonism describes the negative influence that an organism exerts on its interaction partner (Köhl et al., 2019; Weiland-Bräuer, 2021). A biocontrol microorganism (antagonist) can cause the negative effect in different ways that include direct or indirect activity against the pathogen, being the interaction partner. The effect can be achieved by the production of secondary metabolites, which are directly active against pathogens (Köhl et al., 2019; Thambugala et al., 2020). The production of antimicrobial metabolites, known as antibiosis, is a common antagonistic mechanism used by bacterial biocontrol agents against plant pathogens (Elnahal et al., 2022). Next to direct antagonism, indirect mechanisms are applied as well, these include the initiation or enhancement of plant defense towards the pathogen (Köhl et al., 2019). Another example is the competition for nutrition such as iron, which is done via the usage of siderophores for example, these are molecules which are used to harvest iron from the environment, consequently harming the pathogens (Alizadeh et al., 2020; Köhl et al., 2019).

1.4. Microbes in Soil

1.4.1. Actinobacteria

Actinobacteria are the second largest bacterial phylum that prevails in different soil types (Mhete et al., 2020; Mujakić et al., 2022). Moreover, many studies show that these bacteria show antagonistic activities against several plant pathogens in soil, which possibly qualifies them as biocontrol agents (Bubici, 2018; Djebaili, Pellegrini, Bernardi et al., 2021). Actinobacteria are mainly filamentous, gram-positive bacteria, inhabiting ecosystems in soil and water, and hosts such as plants and animals (Barka et al. 2015; Cordovez et al. 2015; Madigan et al. 2019; Jose et al. 2021;). The majority of Actinobacteria inhabit the soil ecosystem, where they are essential in the microbial community (Barka et al., 2015). This phylum is considered the main producer of bioactive compounds and pharmaceuticals, producing about 66% of all antibiotics, antifungals and other bioactive compounds originating from nature (Jose et al., 2021; Siddharth and Vittal, 2018). Among Actinobacteria, *Streptomyces* are the predominant genus in soil and sea ecosystems (Bubici, 2018; Jose et al., 2021). Jose and colleagues (2021) found that *Streptomyces* accounted for 65% of new

compound discoveries among Actinobacteria during the period from 2016-2021. Due to the production of antimicrobial secondary metabolites, *Streptomyces* and other Actinobacteria are also considered possible agents for biological control (Bubici, 2018; Cordovez et al., 2015; Djebaili, Pellegrini, Bernardi et al., 2021; Gebily et al., 2021)

1.4.2. Digestive tracts of millipedes

Millipedes are essential decomposers of organic matter and in the process, these decomposing invertebrates, change structural soil properties and make nutrients available for plants (Glukhova et al., 2018; Griffiths et al., 2021; Pearsons and Tooker, 2021; Schapheer et al., 2021). In the gut of invertebrates such as millipedes a wide range of microorganisms reside and enable them to decompose many different organic materials, these microbial communities also host Actinobacteria. Glukhova and colleagues (2018) regard the digestive system of millipedes as an advisable source for the discovery of new antimicrobials, as this system has hardly been studied yet.

1.5. Secondary metabolites

The products of the secondary metabolism of microbes are organic molecules, which are not essential for vital functions of the producer but are more likely significant for communication and other auxiliary functions in their ecological environment (Siddharth and Vittal, 2018; Tyc et al., 2017). Secondary metabolites of bacteria include a wide variety of chemical compounds that exert different effects on microbes, involving changes in the expression of genes and the behavior of the recipient. Among others, they function as signals for interactions, as growth inhibitors or antibiotics. Based on their chemical and physical properties, secondary metabolites can be divided into soluble and volatile compounds. Many compounds with antibiotic activity were identified in both groups; however, volatile compounds were found to mediate long-distance interactions between microorganisms in soil, this may be beneficial for biocontrol agents (Schulz-Bohm, 2018; Tyc et al., 2017).

1.6. Volatile organic compounds

Microbial volatile compounds are molecules of small size and low molecular weight (< ~300 Da), characterized by a high vapor pressure at room temperature (Lammers et al., 2022; Weisskopf et al., 2021). Many microbial volatiles are formed during primary and secondary metabolism and include chemically distinct classes. These classes can consist of a great variety

of organic compounds such as alcohols, alkanes and terpenes and inorganic compounds like ammonia and hydrogen sulfide for example (Choudoir et al., 2019; Lammers et al., 2022; Schmidt et al., 2015). Many volatiles are nonpolar compounds and contain few functional groups (Lammers et al., 2022). Volatile compounds possess the ability to spread more rapidly in the gas and water phase than soluble compounds, being the first bioactive molecules reaching other microbes (Lammers et al., 2022; Schulz-Bohm, 2018; Weisskopf et al., 2021). These compounds are essential in long-distance interactions of more than 20 cm, functioning as signals and as compounds of defense or attack towards other microbes. Antimicrobial activities mediated by volatile compounds can be found in ecosystems such as soil (Lammers et al., 2022; Tyc et al., 2017; Weisskopf et al., 2021).

1.6.1. Bacterial volatiles with antifungal activity

Bacterial volatiles with antimicrobial activity include among other compounds alcohols, pyrazines and sulfur-containing compounds such as dimethyl disulfide (Lammers et al., 2022; Ossowicki et al., 2017; Tyc et al., 2017). Recent studies showed that many bacterial antimicrobial volatiles and their blends are active against pathogenic fungi (for review see Garbeva and Weisskopf, 2020), their effect manifesting as a negative influence on growth of mycelium and spores (Lammers et al., 2022). *Streptomyces* are known for the production of antimicrobial compounds, especially the abundant production of terpenoids and inorganic volatiles such as ammonia (Lammers et al., 2022). Some examples of antifungal volatiles synthetized by *Streptomyces* species include anisole, butanone, and dimethyl disulfide (Garbeva and Weisskopf, 2020; Lammers et al., 2022).

Recent studies showed antifungal potential of volatile compounds of certain *Streptomyces* species against different fungal phytopathogens (Garbeva and Weisskopf, 2020; Jepsen et al., 2022; Le et al., 2022). Although the antifungal potential of volatiles produced by *Streptomyces* species has been tested against some fungal phytopathogens of the genus *Fusarium* (Garbeva and Weisskopf, 2020), to my knowledge, the effect of solely bacterial volatiles has not been tested on *Geotrichum candidum* yet (Gaete et al. 2022; Ghazanfar et al., 2016; Maldonado et al.,2010). Very few studies have explored the antifungal potential of volatile blends of *Streptomyces* species used in this study, which originate from the digestive tract of millipedes (Danaei et al., 2013; Djebaili, Pellegrini, Ercole et al. 2021; Jepsen et al., 2022).

2. Work aims

The main aim of my BSc project is to screen a collection of soil Actinobacteria isolates for antagonistic activity through volatile compounds against a panel of plant pathogens (Figure 1) and evaluate their potential application as biocontrol agents.

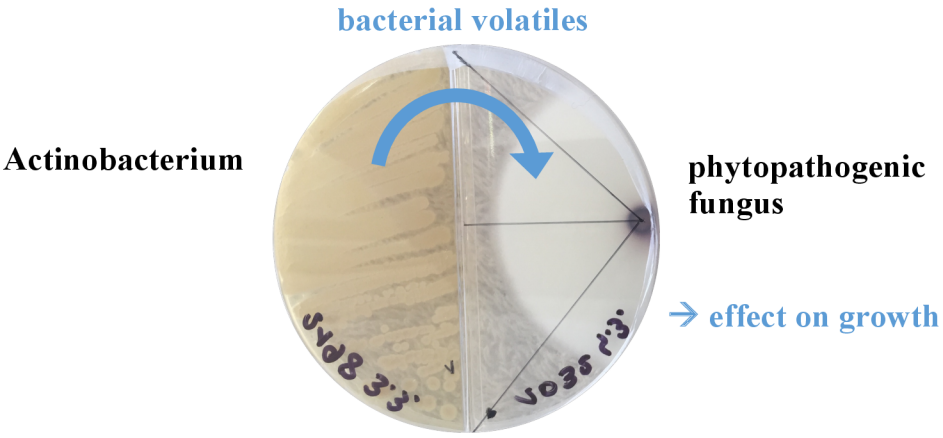


Figure 1: Antagonistic assay as main practical method of this project. Two-compartment Petri dish with volatile producing bacteria in the left and fungal plant pathogens in the right compartment (here *Streptomyces anulatus* and *Fusarium solani*).

3. Materials and methods

3.1. Microorganisms and growth conditions

All used strains, but the model *Streptomyces coelicolor* were received from the collection of the Biology Centre Collections of Organism (BCCO) in České Budějovice. Table 1 shows all bacterial strains which were used in this study, among these, *Streptomyces* species were cultivated on tryptic soy broth (TSB (Panreac), agar 20g (Sigma-Aldrich)) and the *Kutzneria* sp. on M2 (10g L-1 malt extract (Sigma-Aldrich), 4g L-1 yeast extract (Sigma-Aldrich), 4g L-1 glucose (Sigma-Aldrich), 20g L-1 agar (Sigma-Aldrich)). *Streptomyces* (*S.*) species originating from the digestive tract of millipedes were pre-grown for their usage in this study for 1 day at 28°, all other species were pre-grown for 2 days.

Table 1. Strains of Actinobacteria

Strain number from collection	Species	Biological origin
BCCO _10_1627	*Kutzneria* sp.	soil, lowland forest, Mount Cameroon, Cameroon
BCCO_10_ 2198	*Streptomyces anulatus*	millipede gut, Telodeinopus aoiutii, fed on acer
BCCO_10_ 2187	*Streptomyces antibioticus*	millipede gut, Telodeinopus aoutii, fed on quercus
BCCO_10_2169	*Streptomyces hydrogenans*	millipede gut, Telodeinopus aoutii, fed on quercus
Reference strain	*Streptomyces coelicolor* A3(2)	soil (Bentley et al., 2002)

All fungal phytopathogens, which are listed in Table 2, were inoculated on 0.5 potato dextrose agar (19.5 gL-1PDA (Sigma-Aldrich), 10g L-1 agar (Sigma-Aldrich)) at 28°C and pre-grown for two weeks prior to their use in this study.

Table 2. Strains of phytopathogenic fungi

Strain number from collection	Species	Biological origin
BCCO_20_1313	*Geotrichum candidum*	Sokolnica, Czech Republic, vermicompost
BCCO_20_0019	*Fusarium graminearum*	Bavorov, Czech Republic, apple orchard
BCCO_20_0146	*Fusarium oxysporum*	Bavorov, Czech Republic, apple orchard
BCCO_20_1032	*Fusarium solani*	Sokolo brown coal district, Czech Republic, Vilem dump

3.2. Preparation of two-compartment Petri dish system

To evaluate antifungal activity of volatiles of Actinobacteria towards the selected plant pathogens, *Fusarium* spp. (*F.*) and *Geotrichum candidum* (*G. candidum*), two-compartment Petri dishes were used. The bottom plate of these Petri dishes is split into two parts by a separating wall, allowing the usage of two different media and enabling only the transfer of volatiles (Figure 2).

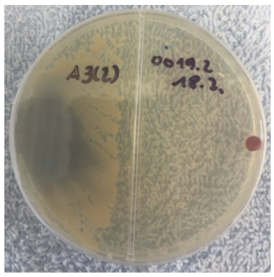


Figure 2. Antifungal volatile assay setup. Two-compartment Petri dish with a pre-grown liquid suspension of Actinobacteria as the volatile producer on the left side and the plug of fungal mycelium of the phytopathogen on the right side.

3.2.1. Preparation of media

Media were prepared as described above and the respective ingredients were weighed and suspended in deionized water. Further, the pH of the solution was adjusted using a pH meter and a base (NaOH) or acid (HCl). The final pH of PDA media was 5.5, and for bacterial media M2 and TSB the pH was adjusted to 6.5. For sterilization, media were autoclaved. The media were poured into two-compartment Petri dishes in a sterilized laminar flow biosafety cabinet. Using 50 ml plastic falcon tubes, 10 ml of the respective medium were transferred to each compartment of the petri-dish.

3.2.2. Inoculation

Liquid suspensions were prepared from each pre-grown bacterial strain. For the preparation of liquid suspensions, 1 ml of sterilized water was added into a 2 ml Eppendorf tube. Subsequently, four large colonies of the isolate were transferred from the pre-grown culture into the Eppendorf tube, using an inoculation loop. These colonies were crushed and mixed pipetting up and down with an automatic pipette. Further the suspended colonies were diluted with 1 ml sterilized water and vortexed for 5 minutes. This procedure was repeated with all bacterial producer strains.

From the liquid suspension of Actinobacteria 100 µl were transferred via an automatic pipette onto the medium for bacteria in one chamber of each two-compartment Petri dish. Further the suspension was spread using a sterile spreader. 12 two-compartment Petri dishes were prepared from each bacterial strain with the above-mentioned procedure, this was repeated with all bacterial producers. Subsequently the plates were incubated at 28°C for two days.

Thereafter a plug of 5 mm in diameter was cut from pre-grown fungal cultures and placed upside down into the other compartment containing 0.5 PDA medium. The plug was placed with the maximum possible distance from the wall, separating this compartment from the other compartment, containing the bacterial volatile producers. Two-compartment Petri dishes, which contained a phytopathogenic fungi in one chamber and only the medium of Actinobacteria within the other, served as negative controls. Each combination of Actinobacteria and fungal plant pathogen and each negative control was prepared in triplicates and all plates were closed with parafilm.

3.3. Incubation and measurements

The plates were placed in the incubator at 28°C for three days, after this period the fungal growth was examined daily for the following five days in measuring the radial growth with a ruler in three directions originating from the plug of mycelium (Figure 3). An exception was the fungal pathogen *Geotrichum candidum* 1313, which was additionally measured after eight days, due to slow growth.

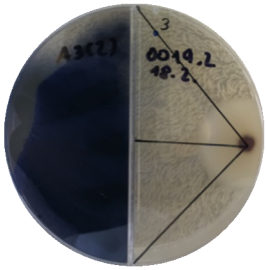


Figure 3. Two-compartment Petri dish at the first day of measurements of the fungal growth. The left side is inoculated with Actinobacteria (here *S. coelicolor* A3(2), staining the medium black) and on the right side the fungus is visible (here *F. graminearum* 0019).

3.4. Data analysis

Student's t-test in R (version 4.1.1) was used to determine statistically significant differences between the growth of each fungus under influence of volatiles compared to the negative control, being the growth of the fungus only influenced by the empty medium of bacteria. For calculations, the mean value of the three measurements of mycelium in all replicates was calculated and the data from the last day before the maximal growth had been reached, was used. To compare the inhibitory potential between volatile producing bacteria, the growth of the fungus under influence of volatiles was transformed to the percentage of growth inhibition. This was done in calculating the difference of fungal growth between control and treatment and in further calculating the relative share of this difference from the normal growth represented by the control.

4. Results

Volatile blends produced by different Actinobacteria were tested for their antifungal potential against four fungal plant pathogens using two-compartment Petri dishes. To test the antifungal activity of *Streptomyces* and *Kutzneria* species, the growth from mycelia of the selected fungal phytopathogens under influence of bacterial volatiles was measured and statistically significant differences to the negative control were evaluated using t-test. The effects on fungal growth and morphology of all volatile blends tested in this study are summarized in Table 3.

Table 3. Summary of effects of volatile blends against the selected phytopathogens over all treatments. Effects on the fungal growth are indicated as follows: "-" depicts a growth inhibition, "+" indicates a growth promotion and "0" indicates no statistically different effect when compared to the control (t-test, $p<0.05$). All values are given in % of growth inhibition when compared to the negative control. The coloration of each table cell indicates the change in morphology of the fungus in response to the bacterial volatiles. A green coloration indicates no visible change when compared to the negative control, grey coloration stands for a decrease in pigmentation and no coloration indicates a complete loss of pigmentation, resulting in white colonies.

fungal phytopathogens (species + strain)	**bacterial volatile producers (species + strain)**				
	S. amulatus 2198	*S. antibioticus* 2187	*S. hydrogenans* 2169	*S. coelicolor* A3(2)	*Kutzneria sp.* 1627
F. solani 1032	-13	-11	0	-15	0
F. graminearum 0019	-8	-7	-12	0	0
F. oxysporum 0146	0	+9	0	0	0
G. candidum 1313	0	0	-10	-20	0

4.1. Inhibitory capacity of *Streptomyces* species

The results indicate that all four *Streptomyces* species exert inhibitory effect on the growth of specific fungal phytopathogens. The hyphal growth reduction varied between about 7 – 20% compared to the control (summarized in Table 3). The strongest inhibition was shown by *S. coelicolor* A3(2) (Figure 4 a) in the interaction with *G. candidum* 1313 (about 20%). Further, volatiles emitted by *S. coelicolor* A3 (2) reduced the hyphal growth of *F. solani* 1032 by

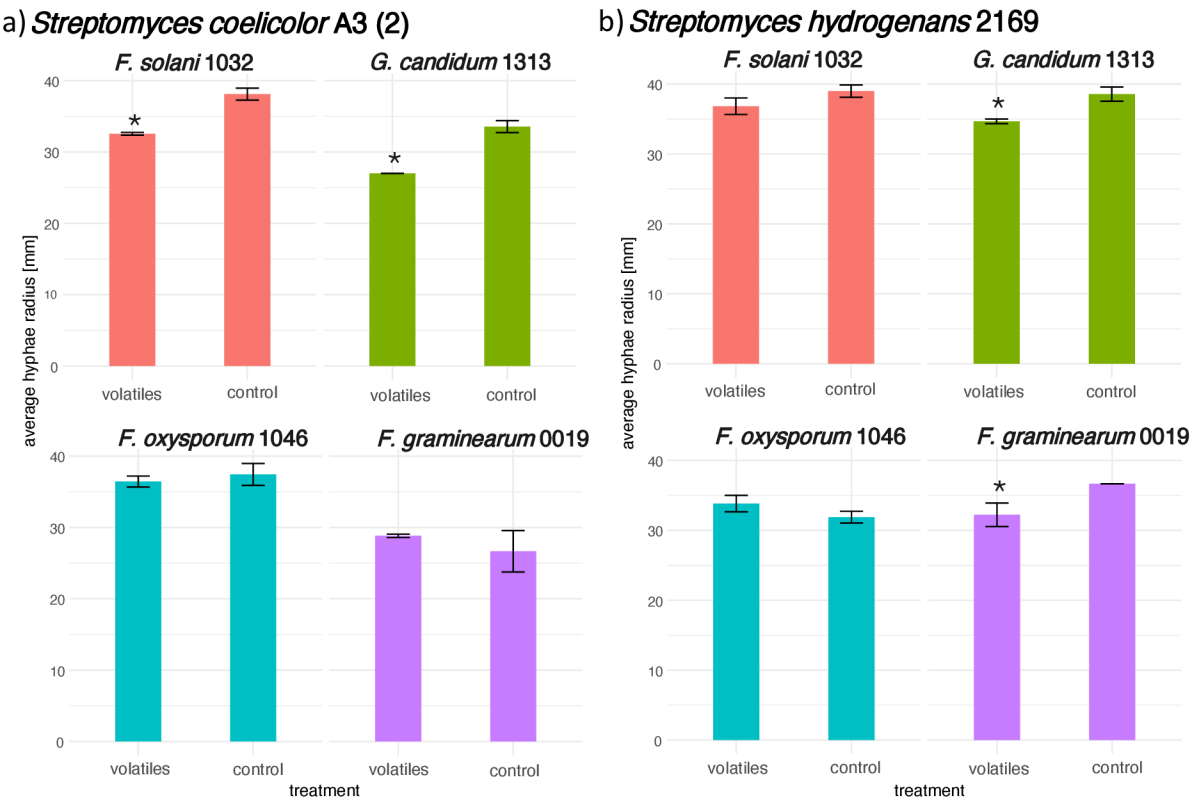
approximately 15%. *F. solani* 1032 was further inhibited by the volatiles of *S. anulatus* 2198 (13%) (Figure 4 c) and *S. antibioticus* 2187 (11%) (Figure 4 d), which both showed their highest inhibitory activity against this pathogen. Among all *Streptomyces* species *S. hydrogenans* 2169 (Figure 4 b) showed the highest inhibitory effect against *F. graminearum* 0019 (about 12%), compared to the inhibition caused by *S. anulatus* 2198 (about 8%) and *S. antibioticus* 2187 (about 7%).

4.1.1. Growth promotion induced by volatiles of *S. antibioticus* 2187

The growth of *F. oxysporum* 0146 was not reduced by volatiles of any bacterial species tested, instead, a growth promotion by approximately 9% due to the volatiles emitted by *S. antibioticus* (Figure 4 d) could be observed. *S. antibioticus* was the only species to significantly promote the growth of a fungal plant pathogen. The volatile blend of this bacterium additionally showed inhibitory capacity against two other *Fusarium* species (*F. graminearum* 0019 and *F. solani* 1032).

4.2. Inhibitory capacity of *Kutzneria* sp. 1627

Exposure of fungal pathogens to volatiles produced by *Kutzneria* sp. 1627 did not cause any significant change in their growth (Figure 4 e).



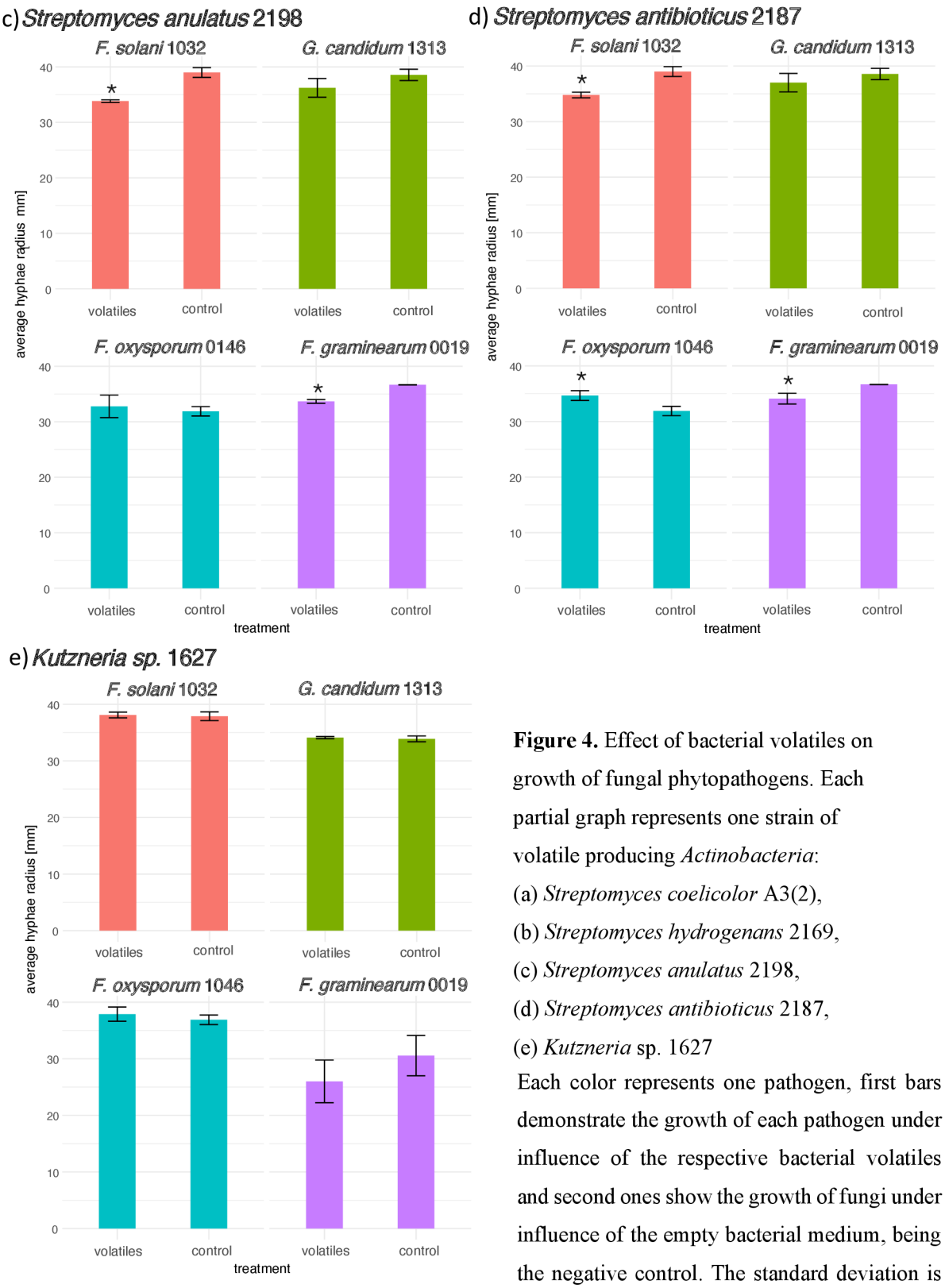


Figure 4. Effect of bacterial volatiles on growth of fungal phytopathogens. Each partial graph represents one strain of volatile producing *Actinobacteria*: (a) *Streptomyces coelicolor* A3(2), (b) *Streptomyces hydrogenans* 2169, (c) *Streptomyces anulatus* 2198, (d) *Streptomyces antibioticus* 2187, (e) *Kutzneria* sp. 1627 Each color represents one pathogen, first bars demonstrate the growth of each pathogen under influence of the respective bacterial volatiles and second ones show the growth of fungi under influence of the empty bacterial medium, being the negative control. The standard deviation is shown in the form of error bars, and the statistically significant differences between treatment with volatiles and negative control are indicated with an asterisk.

4.3. Changes in fungal morphology

Additionally, we found that volatiles of all *Streptomyces* species induced a change in the morphology of all *Fusarium* species but did not affect the morphology of *Geotrichum candidum* 1313 (Table 3). *S. coelicolor* A3 (2) was observed to stop the production of pigments for the most part in all *Fusaria*, resulting in the formation of white colonies (Table 3, Figure 5). Volatiles of *Streptomyces* species, isolated from millipedes, decreased the production of pigments in two Fusaria (*F. graminearum* 0019 and *F. oxysporum* 0146) and totally inhibited the production of pigments of *F. solani* 1032 (Table 3).

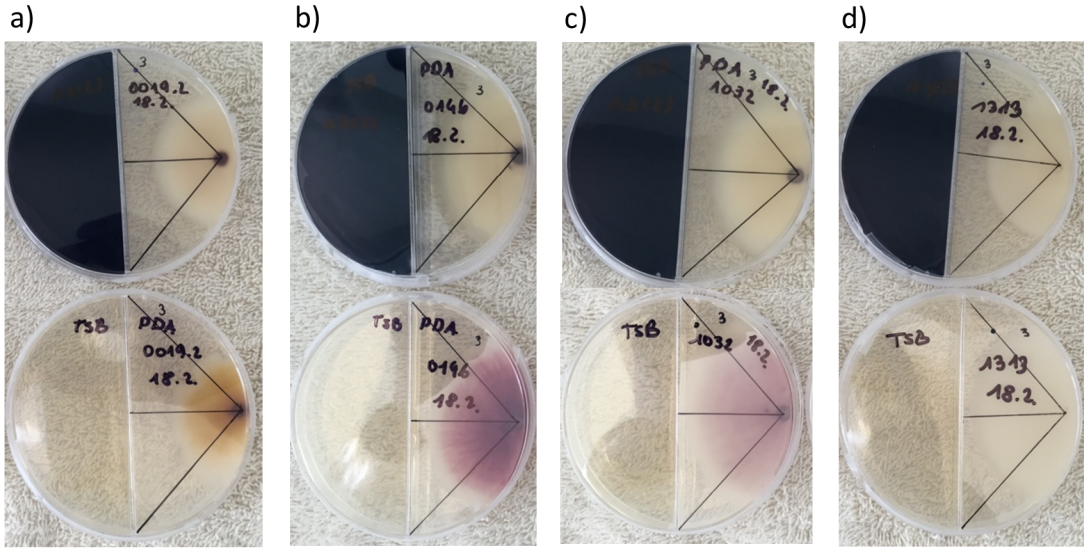


Figure 5. Change in morphology of fungi in response to the volatile blend emitted *by S. coelicolor* A3 (2). Each picture shows the effect of this volatile blend on the morphology of a different phytopathogen: (a) *F. graminearum* 0019, (b) *F. oxysporum* 0146, (c) *F. solani* 1032 and (d) *G. candidum* 1313. The upper row of Petri dishes shows the treatment of the fungus (right compartment) with the volatile blend of *S. coelicolor* A3 (2) (left compartment). The lower row presents the negative control of each fungus (right compartment), these Petri dishes show the fungal morphology not under influence of the bacterial volatiles but under influence of the empty medium.

5. Discussion

In this project we evaluated the antifungal activity of volatiles produced by selected Actinobacteria against fungal plant pathogens belonging to the genus *Fusarium* and *Geotrichum* by performing antagonistic assays with two-compartment Petri dishes. We found that the volatile blends of all four *Streptomyces* species inhibit the growth of specific fungal phytopathogens (*F. solani* 1032, *F. graminearum* 0019, *G. candidum* 1313). These findings are in line with other studies confirming that certain *Streptomyces* species volatiles are active against fungal plant pathogens, including several *Fusarium* species (Garbeva and Weisskopf, 2020). Recent studies which focus on *G. candidum* confirm the growth reducing effect of metabolites produced by different antagonistic bacteria (Gaete et al. 2022; Ghazanfar et al. 2016), which also include a few *Streptomyces* species (Maldonado et al. 2010). Nevertheless, these studies did not focus on volatile metabolites. To my knowledge no volatile compounds of microbial origin have been proven to inhibit the growth of *Geotrichum candidum* yet. Therefore, my study is the first to show it.

Recent studies on the antagonistic activity of volatiles of *Streptomyces* ssp. on *Fusarium* ssp. show ranges of growth inhibition from about 20 to nearly 100%, (Amini, et al. 2016; Corral et al. 2020; Le et al. 2022; Nourozian et al. 2006; Wang et al. 2013; Wu et al. 2015). Compared to these studies, the inhibitory capacity of the *Streptomyces* species investigated here present statistically significant but lower inhibition capacity. Nevertheless, these studies differ in the used methodology, conditions and strains, highlighting the need for more studies which enable a direct comparison.

Our study revealed that volatile blends of the *Kutzneria* sp. 1627 do not influence the growth of the tested fungal phytopathogens. Recent studies provide evidence that different species within this genus produce antifungal metabolites (Devi et al. 2021; Pohanka 2006.; Vijay et al. 2020; Zolova and Garneau-Tsodikova 2014). It has been shown that volatile blends of more related bacterial species, share more similarities, which might explain why the volatiles of the *Kutzneria* sp. 1627 showed a completely different effect on the pathogens (Choudoir et al., 2019; Garbeva and Weisskopf, 2020). Eventually, the different composition of nutrients (M2 medium) compared to the other bacterial producers (*Streptomyces* on TSB) is responsible for the production of a volatile blend which does no longer exert antifungal activity (Weisskopf et al., 2021). It has been suggested that the use of TSB medium probably enables a higher production of sulfur containing volatiles, which

might explain the higher inhibitory potential of volatiles produced by bacteria on this medium (Li et al., 2020).

Interestingly, the strain *Fusarium oxysporum* 0146 was the only fungus which was not inhibited by the volatile blend of any Actinobacteria tested. It appears that this phenomenon can be explained in recent literature, indicating that the response to volatile blends can differ between genera, within them and within the strains of one species (Corral et al., 2020; van Agtmaal et al., 2018; Weisskopf, 2013). This might offer an explanation for the differing responses of the tested species within one genus of *Fusarium* to the volatile blend produced by the same Actinobacterium (*S. antibioticus* 2187). Thus, it might explain why, while supporting the growth of *F. oxysporum* the volatile blend of *S. antibioticus* counteracted the growth of the two other *Fusarium* species. *F. oxysporum* 0146 was the only pathogen, which showed a growth promotion, this was observed when exposed to bacterial volatiles of the *S. antibioticus strain 2187*. To my knowledge the antagonistic effects of the volatiles of *S. antibioticus* on *Fusaria* have not been subject of recent research. We observed a growth promotion of *F. oxysporum* 0146 by the volatiles of *S. antibioticus*. This is in line with recent studies, which provide evidence that bacterial volatiles can have growth inhibitory and growth promoting effect towards microorganisms, such as fungal pathogens in this case (van Agtmaal et al., 2018; Weisskopf et al., 2021). Further this eventually indicates that the volatiles emitted by *S. antibioticus* were used as food source by *F. oxysporum*, this usage of volatiles has been shown by previous research (Briard et al., 2016; Effmert et al., 2012).
Our results are not in line with similar findings of Amini and colleagues (2016), who showed that volatiles of the *Streptomyces* strain KS112, which is closely related to *Streptomyces antibioticus*, could inhibit the growth of *Fusarium oxysporum* f. sp. *ciceris*. While some studies show that *F. oxysporum* species are rather resistant to volatiles produced by *Streptomyces* (Hunziker et al., 2015; Reverchon et al., 2019), others indicate that *Streptomyces* species are able to inhibit *F. oxysporum* (Amini et al., 2016; Wu et al., 2015). The response of this pathogen likely depends on the species used, further van Agtmaal and colleagues (2018) also suggested that the sensitivity of *F. oxysporum* towards bacterial volatiles might depend on the respective strain of this species.

In general, the results of this study confirmed that the production of volatile compounds is specific for each interaction and depends on the involved species, what has been already shown in the literature (Garbeva et al., 2014; van Agtmaal et al., 2018; Weisskopf et al.,

2021). Further the antifungal potential has been shown to differ between similar bacteria in this study, which was also confirmed previously (Garbeva et al., 2014)

The strongest inhibition of approximately 20% was caused by the volatile blend of *Streptomyces coelicolor* A3(2) against *Geotrichum candidum* 1313. Followed by the second highest inhibition of about 15 % against *Fusarium solani 1032*. Different strains of *S. coelicolor* are known to produce antifungal volatiles such as dimethyl disulfide, which has been proven to inhibit the growth of phytopathogenic fungi (Danaei et al., 2013; Jepsen et al., 2022; Wilkins and Schöller, 2009). While reliable information about the sensibility of *G. candidum* towards *Streptomyces* volatiles is scarce, it has been proven that *F. solani* can be inhibited by volatiles of *Streptomyces* species (Alblooshi et al., 2022; Corral et al., 2020; Reverchon et al., 2019). Nevertheless, very few studies addressed the antifungal potential of the volatile blends of different strains of *S. coelicolor* so far (Danaei et al., 2013; Jepsen et al., 2022).
Except for the model *Streptomyces* species *S. coelicolor* A3(2), all other *Streptomyces* species included in this study (*S. anulatus* 2198, *S. antibioticus* 2187, *S. hydrogenans* 2169) originate from the digestive tract of millipedes (Telodeinopus aoiutii). These bacteria have shown inhibitory potential against *Fusarium graminearum* 0019, with *S. hydrogenans* 2169 causing the largest inhibition (11%). Recent studies confirmed that volatiles of specific *Streptomyces* species show antagonistic activity against *F. graminearum* (Le et al., 2022; Nourozian et al., 2006). Further, studies could show that strains of *S. hydrogenans* have antifungal activity against certain phytopathogens, such as some *Fusarium* species (Glukhova et al., 2018; Kaur and Manhas, 2014; Kulkarni et al., 2017). However, to my knowledge, no studies concerning the inhibitory capacity of only volatiles produced by *S. hydrogenans* have been published so far.

Further this study reveals that *Fusarium solani* 1032 was inhibited by *S. anulatus* 2198 (13%) and *S. antibioticus* 2187 (11%). Soltanzadeh and colleagues (2016) showed that isolates similar to *S. antibioticus* were able to inhibit the growth of *F. solani* fsp. *pisi*, using dual culture methods. It has not been confirmed yet that solely volatile compounds of *S. antibioticus* are able to inhibit the growth of *F. solani* (Bubici 2018; Soltanzadeh et al. 2016). Consequently, this study is likely the first to show the antifungal potential of volatiles produced by *S. antibioticus* against *F. solani.* Nevertheless, little is known about the antifungal potential of volatiles of strains of *S. anulatus*, actually to my knowledge there is only one recent study, covering this topic (Djebaili, Pellegrini, Ercole et al. 2021).

These findings suggest the promising antifungal potential of volatiles produced by Streptomyces species inhabiting the digestive tract of millipedes and support recent findings about the antimicrobial potential of Actinobacteria in a similar environment (Glukhova et al., 2018). Due to the fact that millipedes are essential in the decomposition of organic material, and are known to mix soil layers, we may assume that they can contribute to the spreading of these plant beneficial Actinobacteria and by that contribute to general soil health (Glukhova et al., 2018; Griffiths et al., 2021).

Additionally, we found that the tested *Fusaria* responded to the volatiles of *Streptomyces* with a change in their morphology. This became visible in the form of a decrease or total loss of pigmentation among most pathogens, when compared to the control. It has been shown that bacterial volatiles can affect the phenotype of fungi, which was also confirmed for some *Streptomyces* species (Enespa and Chandra, 2017; Ossowicki et al., 2017). Further, sulfur containing volatiles are frequently produced by bacteria and include for example dimethyl disulfide, which shows antifungal activity and can induce changes in morphology of fungi, such as the loss of pigmentation (Lammers et al., 2022; Tyagi et al., 2020; Weisskopf et al., 2021). It has been shown that the red pigment aurofusarin, which is produced by many *Fusaria* sp., is used in the defense against predators (Xu et al., 2019). Therefore, one might speculate that a loss of pigmentation can indicate higher vulnerability of the fungal pathogens. Leading to the assumption that volatiles of *S. coelicolor* A3(2), which largely inhibited the production of pigments (white hyphae) of all *Fusarium* species, had the most pronounced effect on the fungal morphology and physiology and thus eventually on their defense mechanisms. Volatiles of all Actinobacteria, isolated from millipedes showed similar effects towards the morphology of each tested *Fusarium* species, possibly implying that they could share the volatile compounds responsible for these changes in the fungal phenotype.

6. Conclusions and perspectives

Volatile blends of all four tested *Streptomyces* species show inhibitory potential against three out of four of the tested phytopathogens. The model organism *S. coelicolor* A3(2) exhibits the highest inhibitory capacity in this study, possibly qualifying it the most promising biocontrol agent towards *G. candidum*. Further this study proposes the digestive tract of millipedes as possible source for new antifungal volatile organic compounds produced by *Streptomyces* and suggests their possible application in biocontrol of fungal pathogens in agriculture. On the other hand, our results question the biocontrol potential of volatile blends produced by *Kutzneria* sp. against the tested pathogens.

In accordance with Bubici and colleagues (2018), it must be mentioned that there still is the need to investigate the inhibitory potential of *Streptomyces* against the selected phytopathogens. Further research is needed to establish more favoring abiotic conditions for the volatile production of these Actinobacteria to improve the inhibitory capacity. These conditions might include the use of a greater variety of different nutrients, which has been shown to affect the volatile blends produced (Weisskopf et al., 2021). In further studies the identification of the bioactive antifungal volatiles and knowledge about their mechanism of action will be essential to build the basis for the development of efficient biocontrol agents against fungal phytopathogens. During this process it must be considered additionally that the composition of microbial volatile blends also depends on biotic factors such as the interaction with other (micro) organisms and further also on the microbial community (Garbeva and Weisskopf, 2020; Weisskopf et al., 2021).

7. References

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