THE ECOSYSTEM OF SOCIAL SPIDERS AS A SOURCE OF VOLATILE ANTIMICROBIALS

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Illustration by Sandra Markiewicz

For all those spiders
that were more scared of me
than I was of them
all along.

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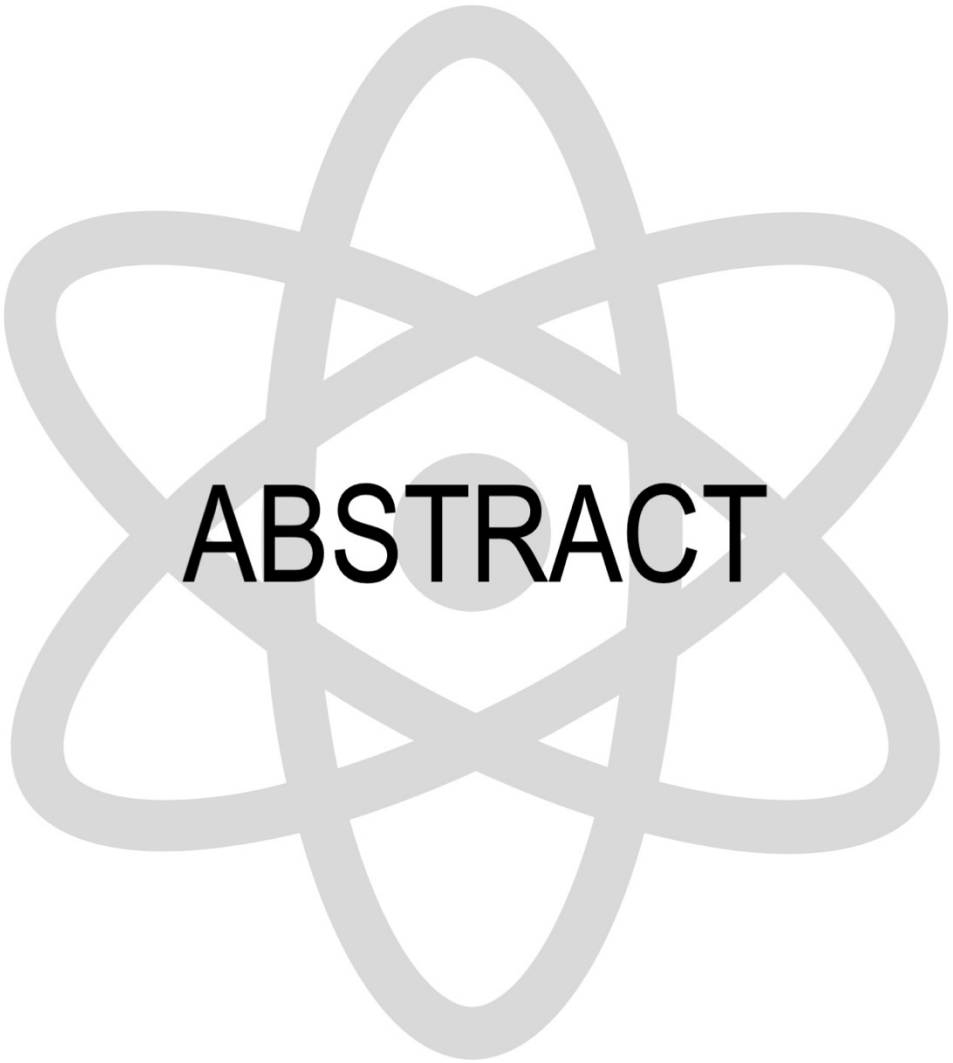
ABBREVIATIONS

B. thuriengiensis	*Bacillus thuriengiensis*
GC	gas chromotagraphy
iChip	isolation chip
m/z	mass-to-charge ratio
PDMS	polydimethylsiloxane
Q-TOF	quadrupole – time-of-flight
S. dumicola	*Stegodyphus dumicola*
sp.	species
SPME	solid-phase microextraction
VOC(s)	volatile organic compound(s)
WHO	World Health Organization

“The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant. Here is a hypothetical illustration.”

(Fleming, 1945)



ABSTRACT

ENGLISH

The discovery of antibiotics around one century ago was a milestone for medicine. However, despite the warning of Alexander Fleming in 1945, antibiotics were used poorly, resulting in many antibiotic-resistant pathogens. Patients infected with resistant pathogens need to get treated with additional antibiotics or, as a last resort, trust completely on their immune system. This causes 700,000 deaths per year. Most clinically used antibiotics have been derived from soil microorganisms, while other niches stayed unexplored. Exploring new niches inhabiting antibiotic-producing microorganisms may result in novel antibiotics. Furthermore, expanding the search from frequently investigated soluble metabolites to volatiles may open up numerous compounds as potential future antibiotics. This thesis is about the search for antimicrobial volatiles produced (among others) by microorganisms from social spider ecosystems, a niche that was little explored until now.

Volatiles are characterized by their high vapor pressure at ambient temperatures, allowing them to distribute fast in both the gas and water phase. They can spread quickly even in complex ecosystems using the air and potentially fulfill functions like communication and antimicrobial defense. Especially, volatiles with antimicrobial activities caught the attention of many scientists because of their potential role in pathogen defense, as we have reviewed (**Article I**). Volatiles are usually produced in the primary metabolism and belong to diverse chemical classes, like hydrocarbons, aromates, alcohols, aldehydes, acids, esters, amides, and thiols. Their antimicrobial spectrum ranges from antifungal, to antibacterial, anti-oomycete, and even broad-spectrum activity. Volatiles are ubiquitously produced. Especially *Bacillus* and *Streptomyces* species are often reported to produce antimicrobial volatiles. Knowledge about antimicrobial volatiles – for example, details about their modes of action – is lacking yet, but these compounds may help to overcome the antimicrobial resistance crisis in the future. Volatiles could be used in medicine and agriculture, either alone or in combination with traditional antibiotics, opening new strategies against antimicrobial resistance.

A promising source of (volatile) antimicrobials is the ecosystem of social arthropods. Due to their lifestyle in dense colonies, they likely spread pathogens between individuals, making antimicrobial defense crucial. Since the presence of antimicrobial volatiles was reported in social insect ecosystems, we investigated the unexplored volatilome of the Namibian social spider *Stegodyphus dumicola* (**Articles II** and **III**). In the first study, we analyzed the *in situ* volatilomes of the spiders' nest, web, and bodies using GC/Q-TOF and revealed that more than 40 % of the tentatively identified volatiles were already known for their antimicrobial activities (**Article II**). We proved the antimicrobial activity of five pure compounds found in the samples, among others against the suggested spider pathogen *Bacillus*

thuringiensis. These results indicate the potential role of antimicrobial volatiles for pathogen defense and could ultimately help explain the spiders' ecological success.

Volatiles from the spider volatilome can originate from various sources, including microorganisms, surrounding plants, the spiders themselves, the spiders' prey, so we analyzed the volatilomes of microbial nest members in a second study. The microbial nest members we selected for this were the bacteria *Massilia* sp. IC2-278, *Massilia* sp. IC2-477, *Sphingomonas* sp. IC-11, and *Streptomyces* sp. IC-207, and the fungus *Aureobasidium* sp. CE_32 (**Article III**). Several volatilomes showed antibacterial and/or antifungal activities against two suggested spider pathogens. The subsequent volatilome analyses using GC/Q-TOF revealed the presence of many volatiles that have already been described as antimicrobials. Five pure volatiles were tested against two suggested spider pathogens, revealing all volatiles as antibacterial, antifungal, or both. These results support the potential role of antimicrobial volatiles in social spider pathogen defense and indicate microbial nest members as the origin of (novel) antimicrobial volatiles.

Together, the articles that constitute this thesis highlight the antimicrobial power of volatiles (**Article I**), indicates the volatilome of the ecosystem of *S. dumicola* as a potential pathogen defense (**Article II**), and finally reveal the spider nest microbiome as a source for antimicrobial volatiles (**Article III**). This knowledge not only adds to the understanding of social spider ecosystems (and likely other social arthropod ecosystems) but also has the potential to open a novel source for antimicrobial compounds that may help to counter the antimicrobial resistance crisis.

DEUTSCH

Die Entdeckung der Antibiotika vor etwa einem Jahrhundert war ein Meilenstein für die Medizin. Doch trotz der Warnung von Alexander Fleming 1945, wurden Antibiotika suboptimal angewendet, was zu zahlreichen antibiotikaresistenten Pathogenen führte. Patienten, die mit resistenten Pathogenen infiziert sind, benötigen zusätzliche Antibiotika, oder müssen im schlimmsten Fall komplett auf das eigene Immunsystem vertrauen. Dies führt jedes Jahr zu 700.000 Toten. Die meisten klinisch genutzten Antibiotika stammen von Mikroorganismen aus dem Erdboden, wohingegen andere Nischen weitestgehend unerforscht geblieben sind. Die Erforschung neuer Nischen, die von Antibiotika-produzierenden Mikroorganismen bewohnt werden, könnte zu neuen Antibiotika führen. Darüber hinaus könnte die Ausweitung der Forschung von häufig untersuchten löslichen Metaboliten zu volatilen Metaboliten zu zahlreichen neuen potentiellen Antibiotika führen. Diese Doktorarbeit handelt von der Suche nach antimikrobiellen Volatilen produziert von (unter anderem) Mikroorganismen aus dem Ökosystem sozialer Spinnen – einer bisher wenig untersuchten Nische.

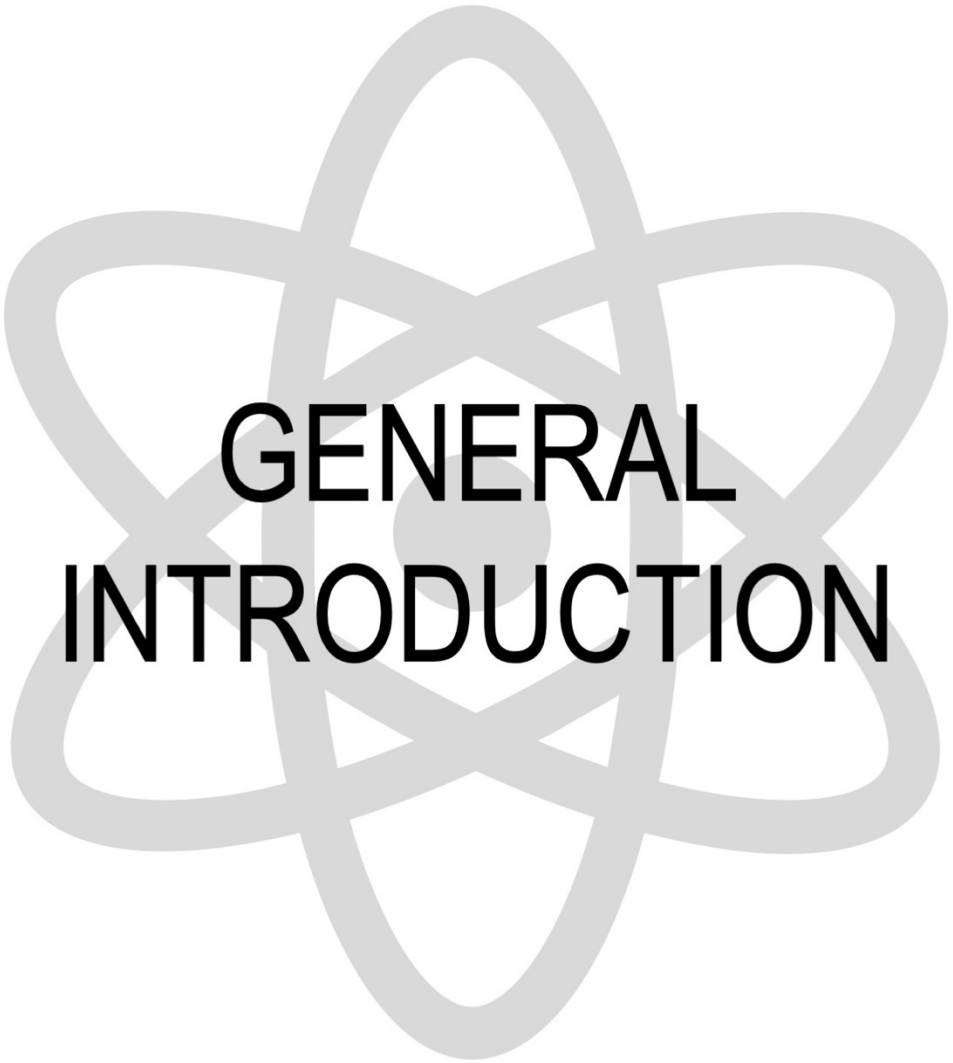
Volatile sind durch ihren hohen Dampfdruck bei Raumtemperatur charakterisiert und können sich sowohl in der Gas- als auch Wasserphase verteilen. Das bedeutet, dass Volatile sich über die Luft auch in komplexen Ökosystemen schnell ausbreiten können, wodurch sie potentiell Aufgaben wie Kommunikation oder Pathogenabwehr ausführen können. Vor allem Volatile mit antimikrobiellen Eigenschaften erregten durch ihre potentielle Rolle in der Pathogenabwehr die Aufmerksamkeit vieler Wissenschaftler*innen, wie wir in einem Übersichtsartikel gezeigt haben (**Artikel I**). Die meisten Volatile werden im Primärstoffwechsel synthetisiert und können diversen chemischen Klassen angehören, wie zum Beispiel Kohlenwasserstoffen, Aromaten, Alkoholen, Aldehyden, Säuren, Estern, Amiden und Thiolen. Ihr antimikrobielles Spektrum reicht von antimykotisch, antibakteriell, antioomykotisch bis hin zu Breitbandwirkungen. Volatile werden ubiquitär synthetisiert, vor allem aber über *Bacillus*- und *Streptomyces*-Arten wird häufig berichtet, dass diese antimikrobielle Volatile produzieren. Unser Wissen über antimikrobielle Volatile – zum Beispiel Details über ihre Wirkmechanismen – ist bisher mangelhaft, jedoch könnten diese Substanzen dabei helfen, die antimikrobielle Resistenzkrise in der Zukunft zu überwinden. Volatile könnten sowohl alleine als auch in Kombination mit herkömmlichen Antibiotika in der Medizin und Landwirtschaft Anwendung finden und dadurch neue Strategien gegen Antibiotikaresistenzen darstellen.

Eine vielversprechende Quelle (volatiler) antimikrobieller Wirkstoffe ist das Ökosystem sozialer Arthropoden. Durch ihren Lebensstil in dicht besiedelten Kolonien sind sie im besonderen Maße gefährdet, Pathogene zwischen Individuen zu verbreiten, wodurch für sie ein antimikrobielles Abwehrsystem entscheidend ist. Da antimikrobielle Volatile bereits in Ökosystemen sozialer Insekten berichtet wurden, haben wir das bisher unerforschte Volatilom

der namibischen sozialen Spinne *Stegodyphus dumicola* untersucht (**Artikel II** und **III**). In der ersten Studie haben wir das *in situ* Volatilom von Spinnennestern, -netzen, und -körpern mittels GC/Q-TOF untersucht und dadurch gezeigt, dass mehr als 40 % der vorläufig identifizierten Volatilen bereits für ihre antimikrobiellen Aktivitäten bekannt waren (**Artikel II**). Wir haben die antimikrobiellen Aktivitäten von fünf reinen Volatilen, die wir in den Proben gefunden haben, unter anderem gegen den vorgeschlagenen Spinnenpathogen *Bacillus thuringiensis* nachgewiesen. Diese Ergebnisse deuten auf eine potentielle Rolle von antimikrobiellen Volatilen zur Pathogenabwehr hin und könnten letztlich dabei helfen, den ökologischen Erfolg sozialer Spinnen besser zu verstehen.

Volatile des Spinnenvolatiloms können aus verschiedensten Quellen stammen, einschließlich Mikroorganismen, umgebenden Pflanzen, den Spinnen selbst oder deren Beute, weshalb wir in einer zweiten Studie die Volatilome von Mikroorganismen untersucht haben, die von den Spinnenestern isoliert wurden. Die ausgewählten Isolate waren die Bakterien *Massilia* sp. IC2-278, *Massilia* sp. IC2-477, *Sphingomonas* sp. IC-11 und *Streptomyces* sp. IC-207, sowie der Pilz *Aureobasidium* sp. CE_32 (**Artikel III**). Mehrere Volatilome waren antibakteriell und/oder antimykotisch gegen die zwei getesteten vorgeschlagene Spinnenpathogene. Die anschließenden Volatilomanalysen mittel GC/Q-TOF zeigten, dass zahlreiche Volatile bereits für ihre antimikrobielle Wirkungen in anderen Studien erwähnt wurden. Fünf reine Volatile wurden gegen die zwei vorgeschlagenen Spinnenpathogene auf ihre antmikrobiellen Aktivitäten getestet, wodurch alle Volatile als antibakteriell, antimykotisch oder sogar beides bestätigt werden konnten. Diese Ergebnisse verdeutlichen die potentielle Rolle antimikrobieller Volatile für die Pathogenabwehr sozialer Spinnen und weisen darauf hin, dass Mikroorganismen aus den Spinnennestern Quelle (neuer) antimikrobieller Volatile sein könnten.

Die Artikel, die diese Doktorarbeit bilden, verdeutlichen das antimikrobielle Vermögen von Volatilen (**Artikel I**), deuten auf eine potentielle Pathogenabwehr durch das Volatilom des *S. dumicola* Ökosystems hin (**Artikel II**), und enthüllen schließlich das Spinnennestmikrobiom als Quelle für antimikrobielle Volatile (**Artikel III**). Dieses Wissen hilft nicht nur, das Ökosystem sozialer Spinnen (und vermutlich auch Ökosysteme anderer sozialer Arthropoden) besser zu verstehen, sondern hat auch das Potential, neue Quellen für antimikrobielle Substanzen zu eröffnen, die dabei helfen könnten, die antimikrobielle Resistenzkrise zu bewältigen.



GENERAL INTRODUCTION

PREAMBLE

They are invisible, though omnipresent. They enable fundamental interactions like communication and antimicrobial defense, though did not receive very much attention for a long time. Volatiles cannot only be used to understand ecological networks better, but might also have the potential to counter the antimicrobial resistance crisis. This thesis is about the search for volatiles with antimicrobial properties. It explains why the nests of social spiders are an especially promising source for antimicrobial volatiles. Ultimately, I will (try to) explain what motivated me to stick my hand into a spiders' nest in the Kalahari Desert.

This thesis is part of the Interdisciplinary Synergy Grant NNF16OC0021110 founded by the Novo Nordisk Foundation. The goal of the project is to establish a novel and innovative source of antimicrobial compounds with the potential to counter the antimicrobial resistance crisis, which is already causing hundreds of thousands of deaths per year. Social spiders are a promising research subject, because their lifestyle makes it highly likely that antimicrobial compounds (of microbial origin) play a crucial role in their defense against pathogens. The project is managed by Trine Bilde (Aarhus University, Denmark), Andreas Schramm (Aarhus University, Denmark), Michael Lalk (University of Greifswald, Germany), and Thomas Vosegaard (Aarhus University, Denmark). Furthermore, for this thesis, we closely collaborated with Paolina Garbeva (NIOO-KNAW, The Netherlands). Within the framework of this thesis, we compiled three publications, one is a review article and two are primary articles.

But first, let's jump back to the previous century, when everything began…

WHAT WAS THE FIRST ANTIBIOTIC DISCOVERED?

The discovery of penicillin was a milestone in medicine, but even milestones can lack attention. When Alexander Fleming first published his discovery of a compound inhibiting staphylococci, in 1929, not many people paid attention (Fleming, 1929). Sixteen years later, Ernst Chain, Alexander Fleming and Howard Florey received a Nobel Prize (The Nobel Prize in Physiology or Medicine 1945). But the complete story of the discovery of antibiotics, influenced by high numbers of wounded during the World Wars, poor civil health care, and infections of scientist's daughters is more complex than it seems at the first glance (Bentley, 2009).

The word antibiotic comes from the Greek words *anti* (ἀντί), meaning "against", and *bios* (βίος), meaning "life". However, today the word describes antibacterial drugs and not compounds being active against life in general (Blair et al., 2015). The first clinically used antibiotic was the synthetic drug Salvarsan (arsphenamine) developed by Paul Ehrlich and clinically introduced in 1910 (**Figure 1A**). The arsenic-based drug was used to treat *Treponema pallidum*, the bacterium causing the sexually transmitted disease syphilis (Gelpi et al., 2015; Hutchings et al., 2019). Later in the 1920s, Gerhard Domagk, employed at the Friedrich Bayer Company (today Bayer AG), and colleagues searched for antibacterial drugs that could be used as pharmaceuticals (Bentley, 2009). After some years of experimental work with *Streptococcus hemolyticus*, they developed the antibacterial sulfamidochrysoidine (Domagk, 1935). In the mid-1930s, it was sold under the trade name Prontosil (**Figure 1B**) and became available internationally. Domagk himself would later review twenty-five years of sulfonamide therapy, including his daughter, who was successfully treated (Domagk, 1957; Bentley, 2009). However, an error in the formulation of Prontosil caused more than a hundred deaths. This was one of the factors contributing to the replacement of Prontosil by what is now probably the most famous antibiotic: Penicillin (Dodds, 2017). Although sulfonamides are still in use.

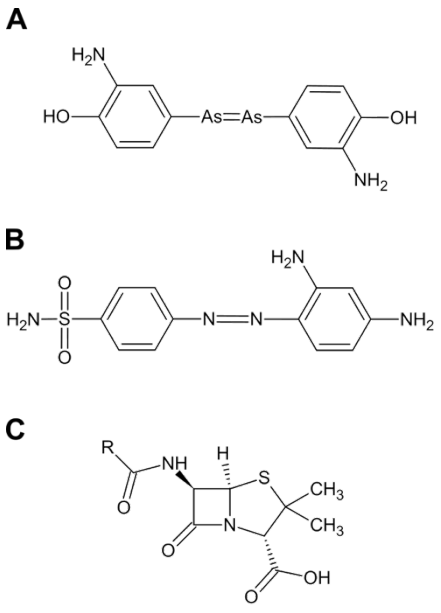


Figure 1 | Chemical structures of Salvarsan (arsphenamine) (A), Prontosil (sulfamidochrysoidine) (B), and Penicillin (C).

In 1928, Alexander Fleming recognized a fungal infection of a Petri dish while experimenting with staphylococci. He understood that the observed bacterial lysis around the fungal growth must be caused by an antibiotic. He named the novel compound after its producer *Penicillium rubrum* (Bentley, 2009). Luckily, he used the genus name, because the species name would later turn out to be wrong. And so, the story of penicillin began. Even though there were some clinical successes in treating infections using the newly discovered compound, Fleming did not manage to make the necessary progress to get enough attention and support to develop penicillin into a novel drug. It would take four years from Fleming's first publication until the chemist Harold Raistrick and his colleagues developed an interest in penicillin and correctly identified the name of its producer, the fungus *Penicillium notatum* (Bentley, 2009). But Raistrick gave up on penicillin as a research topic because of its instability and unsuitability as PhD project for his students (Birkinshaw, 1972). Penicillin got forgotten for some years again. In the late-1930s, Howard Florey, Norman Heatley, and Ernst Boris Chain managed to produce a more stable and purified form of penicillin (**Figure 1C**) (Abraham et al., 1941). It is not delivered in detail what drove them to investigate penicillin, but the successful treatment of Florey's daughter with Prontosil in 1936 might have motivated him to put more effort into novel compounds for chemotherapy (Bentley, 2009). However, Florey and Heatley induced the upscaling of the penicillin production in North America. This was the start of large-scale antibiotic production and a milestone in the fermentation industry (Bentley and Bennett, 2008). In 1945, Dorothy Hodgkin solved the beta-lactam structure of penicillin, paving the way for the production of semi-synthetic derivates (Hutchings et al., 2019). In the same year, Fleming, Chain, and Florey jointly got the Nobel Prize for Physiology or Medicine "for the discovery of penicillin and its curative effect in various infectious diseases" (The Nobel Prize in Physiology or Medicine 1945). Interestingly, Fleming warned in his Nobel Prize lecture, *"The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."* (Fleming, 1945)

After the success of penicillin, scientists understood the potential of microorganisms for antibiotic production (Salvarsan and Prontosil were both synthetic) and started systemic screenings for those. Selman Waksman worked intensively with actinomycetes and identified the genus *Streptomyces* as a promising producer of bioactive metabolites (Hutchings et al., 2019). Indeed, actinomycetes are still the source for the majority of antibiotics today (Hopwood, 2008). With the work of Waksman and his colleagues, the "Golden Age"

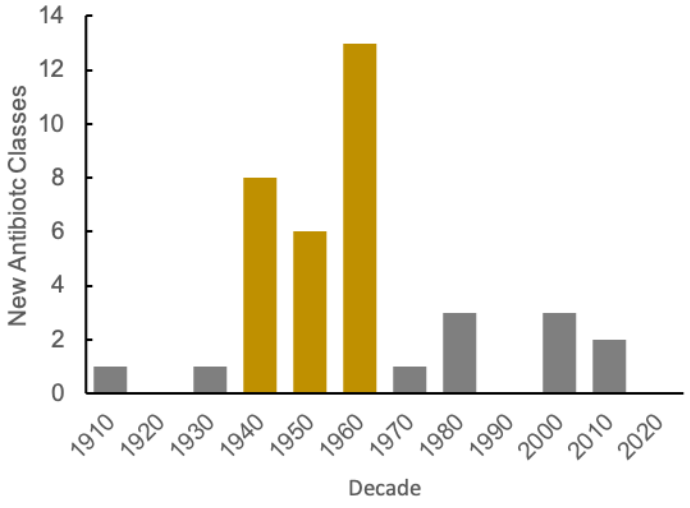


Figure 2 | Number of newly-clinical introduced antibiotic classes per decade. The colored bars indicate the "Golden Age" of antibiotics. Numbers are based on (Hutchings et al., 2019).

of antibiotic discovery began and in the 1940s, ‘50s, and ‘60s, more than twenty novel classes of antibiotics were discovered (**Figure 2**). But since the 1970s, only a few novel classes were discovered. In fact, most antibiotics in clinical trials today are rather derivates of old ones than novel classes (Katz and Baltz, 2016). Since the turn of the millennium, only five novel classes of antibiotics were clinically introduced (Hutchings et al., 2019). Why the lack of novel antibiotic classes is an global issue and which other reasons led to the antimicrobial resistance crisis I will discuss in the next chapter.

WHAT IS THE ANTIMICROBIAL RESISTANCE CRISIS?

The frequent use of antibiotics has resulted in an increase of bacterial resistances against specific antibiotics. Patients infected with resistant bacteria need treatments with additional antibiotics or, in the last resort, depend completely their immune system. This global issue is called “antimicrobial resistance crisis”. The crisis leads to 700,000 deaths per year and by 2050 it might be 10 million (O’Neill, 2016). According to the World Health Organization (WHO), approximately 30 classes of antibiotics that are clinically used in human medicine are “critically important” (WHO, 2016). The WHO (2018, 2019, 2020) and the European Centre for Disease Prevention and Control (2020b, 2020, 2020a) frequently publish reports on antibiotic consumption and resistance. I would like to highlight two key messages here. First, the important and highly virulent bacterial pathogens with antimicrobial resistances are *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter* species, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Streptococcus pneumoniae*, often grouped together with the acronym “ESKAPE”. Second, in some countries, more than 50 % of the reported pathogens are resistant to certain antibiotics (e.g., *Klebsiella pneumoniae* to carbapenems in several south-western European countries). This illustrates the urgency to counter the antimicrobial resistance crisis.

The first resistance to Salvarsan was reported in the late 1920s, and more reports followed soon (Hutchings et al., 2019). The way antibiotic resistance is reported has often led people to believe that antibiotic resistance is a relatively modern phenomenon. In fact, genes encoding resistance to various classes of antibiotics were found in around 30,000 years-old permafrost soil in Yukon, Canada, clearly indicating that antibiotic resistance is an ancient, natural phenomenon (D’Costa et al., 2011). However, the way people used antibiotics in the last decades led to the accumulation of resistant pathogens. The causes of the resulting antimicrobial resistance crisis are as diverse as complex (Michael et al., 2014; Dadgostar, 2019; Shami, 2020).

Globalization is often beneficial, but where people travel, pathogens travel as well (Castro-Sánchez et al., 2016). These days, it is possible to reach nearly every location on earth within one or two days. This is shorter than many pathogen’s incubation time and allows a quick and uncontrolled spread of pathogens (Michael et al., 2014). In addition, urbanization results in high population densities, which increases the risk of spreading pathogens among individuals. Today, more than half of the world’s population lives within cities (Michael et al., 2014). I will come back to that issue when talking about social spiders, later.

Private use of antibiotics without prescription by a doctor (self-medication) frequently results in poor or completely wrong treatments. Poor treatment of pathogens, for example too few doses, may result in an incomplete suppression of pathogens causing the selection of

relatively resistant ones (Cals et al., 2008). A completely wrong treatment is for example the use of antibiotics when infected with a virus. The antibiotic is not effective at all against the virus but still comes with side effects like influencing the (beneficial) microbiome and supporting the development of resistance which can ultimately get transferred to pathogenic bacteria (Michael et al., 2014). In many countries, access to antibiotics is not effectively regulated, and even in those where it is, hoarding of leftover antibiotics from previous treatments makes self-medications easy (Michael et al., 2014). Furthermore, numerous commercials for cleaning supplies often give the wrong impression that microorganisms are disadvantageous in general. In fact, removing microorganisms from all surfaces and our bodies is neither possible nor beneficial. On the contrary, exposure to microorganisms improves the human immune systems (Maslowski and Mackay, 2011).

The clinical use of antibiotics also contributes to the development of antimicrobial resistances. Ideally, the pathogen would be identified before antibiotic prescription so an appropriate antibiotic could be selected. However, due to traditional practices based on experience, the urgent need to treat patients in life-threatening situations, and the patient's expectation of immediate treatment, antibiotics are often unnecessarily used (McNulty et al., 2007; Michael et al., 2014). Furthermore, patients with weak immune systems sometimes need prophylactic treatment, for example, to avoid post-operative wound infections (Shami, 2020). Finally, economical interest of hospitals and cooperating pharmaceutical companies can cause over-prescriptions of antibiotics (Dadgostar, 2019).

Antibiotics are not only used in human medicine, but also in agriculture. A study reports that approximately 80 % of the antibiotics in the USA are fed to livestock (Bartlett et al., 2013). The antibiotics are used prophylactically and increase the productivity of livestock (Hao et al., 2014). For example, the overuse of virginiamycin used to promote growth and prevent infections, can lead to streptogramin resistant bacteria, and is therefore banned in Europe (Shami, 2020).

Finally, within a capitalistic market, it is very unattractive to invest in novel antibiotics. Despite scientific challenges, the legal regulations make it expensive and risky to develop antibiotics and the financial output is small. That is why many would argue that the antimicrobial resistance crisis needs to be countered with help of global funds rather than trust profit-orientated companies (O'Neill, 2016).

Regardless of financial argumentations, there are several approaches to overcome the lack of novel antibiotics and ultimately solve the antimicrobial resistance crisis (Czaplewski et al., 2016). For example, bacterial infections could be treated with bacteriophages that infect and kill the pathogens. The bacteriophages could be applied in small doses because they replicate using their host cells (Hua et al., 2014; Czaplewski et al., 2016). Interestingly, bacteriophages have actually been used as a treatment for bacterial infections before, but were replaced by antibiotics (Gordillo Altamirano and Barr). Other approaches are based on prevention instead of treatments. Another approach is the prevention of bacterial infections with the use of vaccines, which are already very well established (Czaplewski et al., 2016). It is also under consideration that antibodies could be developed that bind specifically to pathogens or their toxins and inactivate them in case of infection (Hua et al., 2014). However, finding novel antibiotics is one of the most feasible approaches due to their successful use for

decades and available development pipelines. In the next chapter, I will discuss why especially microorganisms are a promising source for novel classes of antibiotics.

WHY DO SO MANY MICROORGANISMS PRODUCE ANTIBIOTICS?

The most common theory is that microorganisms produce antibiotics to compete with other microorganisms for nutrients (Challinor and Bode, 2015; Traxler and Kolter, 2015; Hutchings et al., 2019). In fact, the nutrient limitation itself often activates antibiotic production. This seems illogical, considering that antibiotic production is very costly (Traxler and Kolter, 2015). To understand this, it is important to distinguish between direct and indirect competition. If the biosynthesis of antibiotics would be triggered by direct competition microorganisms in the soil would probably be triggered to produce antibiotics continuously, due to the high density of up to 10^9 bacteria, 10^6 fungi, and numerous other (micro)organisms in one gram of soil (Young and Crawford, 2014). Instead, antibiotic production is often activated when the microorganisms experience stress, indicating indirect competition as a trigger. This is called “competition sensing” (Cornforth and Foster, 2013). This makes it possible for microorganisms to recognize a situation of actual stress (such as nutrient limitation) and to distinguish this situation from other types of exposure to other microorganisms.

Interestingly, microbial antibiotics have been also suggested as signaling molecules due to the observation that concentrations in natural environments are often sub-inhibitory (Yim et al., 2006; Hutchings et al., 2019). But even though many studies indicate chemically-mediated interactions between bacteria based on antibiotics, there is no proof of ecological functions as signaling molecules. Additionally, the function as signaling molecule could just be a side-effect of the antibiotic activity (Traxler and Kolter, 2015).

Penicillin as the first microbial antibiotic to be discovered, was produced by a fungus, *Penicillium notatum.* Conversely, most of the antibiotics that are in use today are produced by bacteria, most notably those of the genus *Streptomyces,* belonging to the class of Actinobacteria (Actinomycetes) (Watve et al., 2001; Bentley, 2009; Hutchings et al., 2019). For example, streptomycin, produced by *Streptomyces griseus*, was the first antibiotic used to treat the tuberculosis bacterium *Mycobacterium tuberculosis* (Daniel, 2006). Likewise, daptomycin, one of the newest antibiotics, is produced by *Streptomyces roseosporus* (LaPlante and Rybak, 2004). However, other bacterial classes also produce antibiotics. For example, *Bacillus subtilis* produces bacitracin A (Hutchings et al., 2019).

Microorganisms are clearly highly potent antibiotic producers. Nevertheless, the “Golden Age” of antibiotic discovery has ended around half a century ago. A common explanation for the subsequent lack of novel classes is that the “low hanging fruits” have been picked, although the number of undiscovered antibiotics is hard to predict (Yim et al., 2006). There are two promising strategies to discover new antibiotics: (I) discovery of new niches inhabiting antibiotic-producing microorganisms and (II) finding new methods to cultivate

uncultured microorganisms. Both strategies were combined within the project this thesis contributes.

Today, most clinically used antibiotics are produced by bacteria inhabiting soil. By investigating other niches new microorganisms with the potential to produce antibiotics could be found. For example, Hutchings and colleagues suggest investigating marine environments (Hutchings et al., 2019). Scientists discovered that the marine bacterium *Salinispora tropica* produces an anti-cancer compound, salinosporamide A (Gulder and Moore, 2010). Another new niche could be the human microbiome due to findings like lugdunin, a novel peptide antibiotic produced by the nasal bacterium *Staphylococcus lugdunensis* inhibiting *Staphylococcus aureus* (Zipperer et al., 2016). In the present thesis, we investigated the microbiome of Namibian social spiders and established a new niche with a high potential to find new antibiotics as we will see later in **Articles II** and **III**. In addition to discovering new niches, new cultivation methods could help to find potent microorganisms. With classical isolation methods, the majority of microorganisms cannot be cultivated *in vitro* due to too high or too low levels of nutrients or symbiosis partners. This is known as the “great plate count anomaly” (Staley and Konopka, 1985). Methods like isolation chip (iChip) can enable the cultivation of microorganisms that could not be cultivated using classical methods. The device separates single cells from each other, so that each species present in a mixture of microorganisms can be cultivated in its own *in situ* environment (Nichols et al., 2010). Our project partner from Aarhus University used a variation on this method to isolate microorganisms from the social spider nests (Nazipi et al., submitted) we investigated for their antimicrobial potential (**Article III)**.

HOW DO ANTIBIOTICS WORK?

Most antibiotics work by inhibiting critical cell processes: DNA replication, RNA synthesis, cell wall synthesis, and protein synthesis (Kohanski et al., 2010). In this chapter, I will briefly summarize the mode of action of antibiotics. The quinolone class of antibiotics inhibits DNA replication by interfering with topoisomerase II (also called DNA gyrase) and topoisomerase IV (Drlica et al., 2008). Topoisomerases facilitate the negative and positive supercoiling of DNA. For example, topoisomerase II negatively supercoils (unwinds) DNA strands to prepare them for processes like replication for which they need to cleave and reconnect both strands. Quinolone antibiotics bind covalently to topoisomerases and block them after DNA strand cleavage causing an immediate inhibition of DNA synthesis which is bacteriostatic (Drlica et al., 2008). It is important to notice that the quinolones used in medicine inhibit prokaryotic topoisomerases much more than eukaryotic ones making them usable as pathogen treatment in humans (Kohanski et al., 2010).

Rifamycins, which belong to the ansamycins, inhibit RNA synthesis by blocking the RNA polymerase. The antibiotic binds the enzyme with high affinity and prohibits transcription (Floss and Yu, 2005). Rifamycins are bactericidal to Gram-positive and bacteriostatic to Gram-negative bacteria, due to differences in drug uptake. Rifamycins are often used in combination with other antibiotics because resistance against them develops fast (Wehrli, 1983).

A likewise fundamental cell process is protein synthesis which is targeted by several classes of antibiotics. The bacterial ribosome, which is the site of protein biosynthesis, is divided into two subunits, namely 50S and 30S. Whereas macrolides, lincosamides, streptogramins, amphenicols, and oxazolidinones inhibit the 50S subunit, tetracyclines and aminocyclitols inhibit the 30S subunit (Mukhtar and Wright, 2005). The exact modes of action differ between the antibiotic classes and are reviewed elsewhere (Kohanski et al., 2010).

Another effective target of antibiotics is the bacterial cell wall, because of its importance for cell stability and viability. The bacterial cell wall is composed out of polysaccharide strands (altering *N*-acetylglucosamine and *N*-acetylmuramic acid) which are covalently cross-linked by peptides forming peptidoglycans (Dörr et al., 2019). The crosslinks between the peptidoglycans are formed between a diaminopimelic acid from one peptide and a D-alanine from the other which is realized by the transpeptidase (Dörr et al., 2019). The antibiotics belonging to the β-lactams (e.g. penicillins, carbapenems, cephalosporins) act as analogs to the peptides of the peptidoglycans, blocking the enzymatic reaction, and ultimately inhibit the transpeptidase (Waxman et al., 1980). In contrast, the antibiotic class of glycopeptides binds to the peptidoglycans which block the transpeptidase activity (Kahne et al., 2005). While β-lactam antibiotics are effective against both Gram-positive and Gram-

negative bacteria, glycopeptide antibiotics are only effective against Gram-positive bacteria (Kohanski et al., 2010).

It is important to note that the actual effectiveness of antibiotics cannot be generalized. For example, penicillin can be used against both Gram-positive and Gram-negative bacteria, but is more effective against Gram-positive bacteria because the outer membrane of Gram-negative bacteria functions as barrier (Soares et al., 2012). Antibiotics that inhibit the bacterial ribosome target variable regions of the ribosomes (proteins and RNA) making them species-specific (Roberts et al., 2008). In addition, the combination of antibiotics may influence their effectivity in additive, synergistic, or antagonistic ways. Additive means the combination results in an equal effect compared to the sum of the combined antibiotics, synergistic means an increased effect, and antagonistic means a decreased effect (Kohanski et al., 2010). However, the best treatments of pathogens cannot be generalized and should be done by physicians.

HOW DOES ANTIBIOTIC RESISTANCE WORK?

The resistance mechanisms can be categorized into four main groups: 1) avoiding the entry of antibiotics by spatial exclusion, 2) avoiding the accumulation by antibiotic modification, 3) avoiding the binding by target modification, and 4) avoiding the toxicity by bypassing pathways (Blair et al., 2015; Yelin and Kishony, 2018).

Gram-negative bacteria have an advantage over Gram-positive ones regarding antibiotic resistance due to their outer membrane that forms a permeability barrier. While hydrophobic antibiotics can pass the cell membrane, hydrophilic antibiotics depend on porins to cross the cell membrane (Vargiu and Nikaido, 2012). The permeability of the outer membrane can be reduced by changes in its lipid composition, by reducing the number of porins, or by producing porins with higher selectivity (Delcour, 2009; Blair et al., 2015). If antibiotics do make it across the membrane, efflux pumps may actively transport antibiotics out of the cell. Many of such pumps are unspecific, which is why they are called multidrug resistance efflux pumps (Floyd et al., 2010). Interestingly, efflux pumps may be encoded on plasmids, making them exchangeable via horizontal gene transfer (Dolejska et al., 2013).

If an antibiotic has entered the bacterial cell, they may be inactivated by hydrolysis or steric hindrance. For example, some bacteria produce β-lactamases, enzymes that degrade β-lactam antibiotics resulting in their inactivation (Abraham and Chain, 1940). Similar enzymes may evolve that inactivate new antibiotic classes, like extended-spectrum β-lactamases (Shah et al., 2004). Other enzymes cause steric hindrance by adding substitutes (e.g., acyl, phosphate, nucleotidyl, and ribitoyl groups) to the antibiotics. As a result, the antibiotics lose their ability to bind to their targets (Wright, 2005).

Next to modifying the antibiotic, the molecular target itself might be modified preventing the antibiotic from binding. For example, linezolid, an oxazolidinone antibiotic, targets the ribosomal 23S rRNA (a component of the 50S subunit) of Gram-positive bacteria. The 23S rRNA is encoded by multiple copies whereas some are mutated causing ultimately the selection of resistant strains (Billal et al., 2011). Furthermore, the antibiotic targets may get protected by modifications that do not require mutations. For example, the 23S rRNA from the example just now can be methylated by the chloramphenicol-florfenicol resistance methyltransferase causing resistance to several antibiotics including oxazolidinones (Long et al., 2006).

Finally, bypassing a metabolic pathway can make the antibiotic target redundant, for example when the bacterium shifts from respiratory to fermentative metabolism of glucose (Zampieri et al., 2017). The antibiotic may still bind to its target but is no longer toxic to the bacterial cell.

WHAT MAKES VOLATILES INNOVATIVE ANTIMICROBIALS? (ARTICLE I)

In recent years, volatiles have been reported more and more often for their antimicrobial activities, making them an innovative and promising approach to counter the antimicrobial resistance crisis. Thus, we reviewed the antimicrobial potential of volatiles (**Article I**), which will be summarized in this chapter. Although the review article is focused on volatiles of bacterial origin, most of the information applies to all volatiles.

Volatiles (sometimes referred to as volatile organic compounds, VOCs) are characterized by their high vapor pressure at ambient temperatures (Rowan, 2011). This is due to their low molecular weights (maximum of 200-500 Dalton), low boiling points, and often lipophilic moieties (Rowan, 2011; Schmidt et al., 2015; Schulz-Bohm et al., 2017). Ultimately, these characteristics engender that volatiles do not depend on solvents. Volatiles can still spread fast in both, the gas phase and water phase (Weisskopf et al., 2021) making them suitable for functions like communication and microbial defense, even in complex ecosystems like soil or insect nests (**Article II**; Chen et al., 1998; Garbeva et al., 2014; Ossowicki et al., 2017; Li et al., 2020).

The biosynthesis of volatiles usually takes place within the primary metabolism, starting with pyruvate (**Figure 3**) (Peñuelas et al., 2014; Schmidt et al., 2015). Pyruvate can get fermented to alcohols and acids under anaerobic conditions or metabolized to acetyl-CoA under aerobic conditions (Peñuelas et al., 2014). Acetyl-CoA can enter the fatty acid metabolism, typically resulting in aldehydes, ketones, and fatty acids (Weisskopf et al., 2021). Alternatively, acetyl-CoA can enter the citric acid cycle and get metabolized to amino acids, which are the precursors of most aromatic, S-containing, and N-containing volatiles (Schmidt et al., 2015). Yet another important group of volatiles are the terpenes, which can be metabolized starting from pyruvate through the deoxyxylose pathway or starting from Acetyl-CoA through the mevalonate pathway (Weisskopf et al., 2021).

The antimicrobial activities of volatiles are as diverse as their biochemical pathways, including antibacterial, antifungal, and anti-oomycete to broad-spectrum antimicrobial activities (Rybakova et al., 2017; Bruisson et al., 2019; Janssens et al., 2019; Garrido et al., 2020). Especially, S-containing volatiles often have strong antimicrobial activities. For example, dimethyl disulfide is commonly produced by bacteria and was developed in the fumigant PALADIN® to target soil-borne plant pathogens (Schulz-Bohm et al., 2017). In **Article I** we provided an overview table showing the antimicrobial activities of more than 40 bacteria. Even though most microorganisms produce volatiles, it is remarkable how many antimicrobial volatiles are produced by especially *Bacillus* and *Streptomyces* species. For example, the volatiles produced by *Bacillus amyloliquefaciens* were shown to be both antibacterial and antifungal (Lee et al., 2017; Wu et al., 2019). *Streptomyces* is already known

as a very potent antibiotic producer which is why many studies investigated antimicrobial volatiles. Furthermore, some studies indicate that the co-cultivation of bacteria can change their volatile profile as well as their antimicrobial activity (Song et al., 2015; Tyc et al., 2017). In contrast to clinically used antibiotics, volatiles often have unknown modes of action. However, some studies indicate influences on quorum sensing, DNA, and cell walls (Chernin et al., 2011; Xing et al., 2018; Janssens et al., 2019).

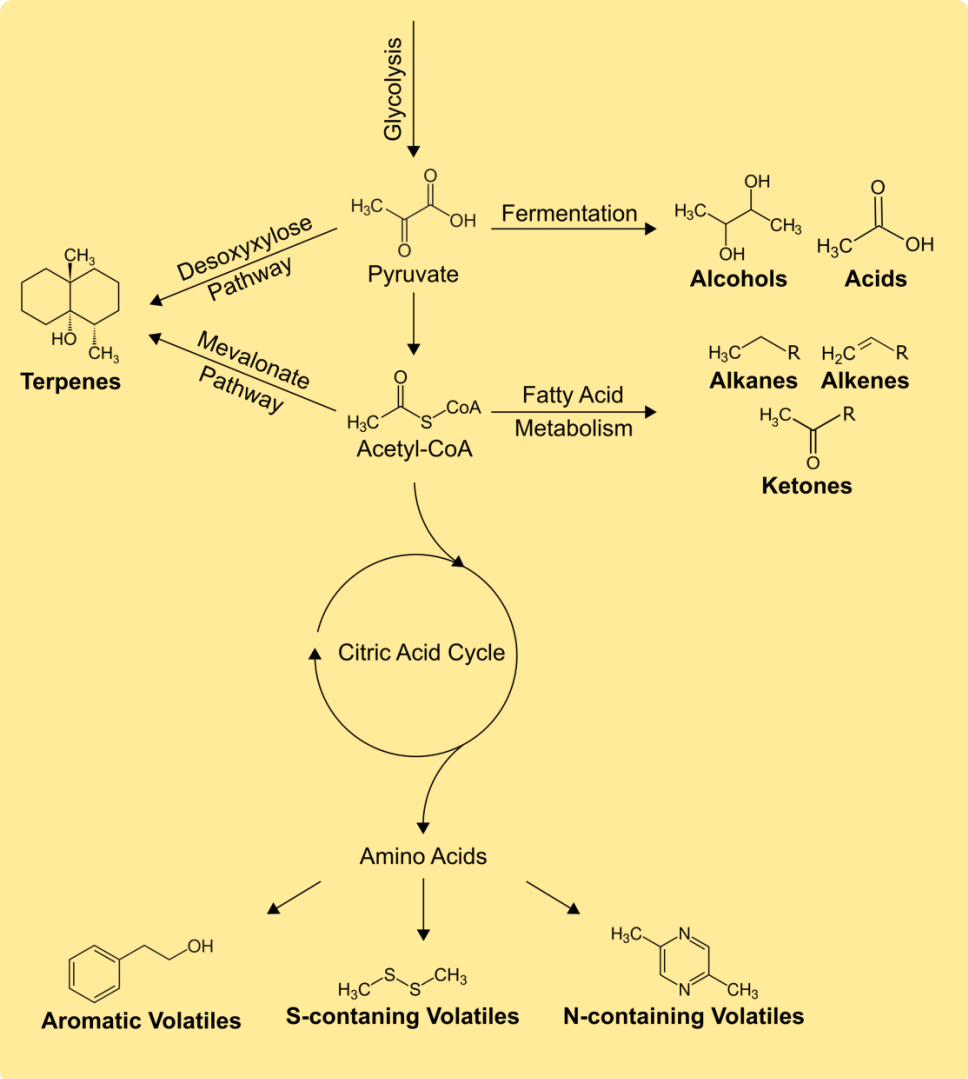


Figure 3 | Overview of main biochemical pathways for the production of bacterial volatiles. The chemical structures show representative examples: alcohols (2,3-butanediol), acids (acetoin), alkanes (general structure), alkenes (general structure), ketones (general structure), terpenes (geosmin), aromatic volatiles (2-phenylethanol), S-containing volatiles (dimethyl disulfide) and N-containing volatiles (2,5-dimethylpyrazine). This is Figure 1 from **Article I** (modified).

Several methods to test the antimicrobial activity of volatiles were developed and can be categorized into two main groups: direct and indirect methods. In indirect methods, the volatiles need to diffuse through the gas phase to reach their target, whereas in direct methods they diffuse through a solvent. For this thesis, we used the two-chamber Petri dish approach as an indirect method and the agar diffusion approach as a direct method (**Articles II** and **III**).

In short, volatiles are a diverse group of chemical compounds that are produced commonly by microorganisms and have often antimicrobial activities. This makes them an

exciting research topic in ecology as well as pharmacy, and potentially usefull to counter the antimicrobial resistance crisis. Volatiles could eventually be developed into antibiotics, which could be used alone or in combination with common antibiotics (Avalos et al., 2019). Furthermore, volatiles can be used as biomarkers to detect pathogens in a fast and reliable way, which could reduce the need to use antibiotics (Traxler et al., 2018).

HOW ARE VOLATILES ANALYZED?

Here, I will briefly describe the main methods for volatile analysis: gas chromatography (GC) (Lu et al., 2017). This chapter gives an overview of the techniques used within this thesis, but the principle of GC is universal. Methodical details are skipped and can be found in **Articles II** and **III**.

The first step in volatilomics (the study of volatiles) is sampling, which is often referred to as “trapping” because the volatiles need to be immobilized from the headspace (gas phase around a sample) (Green, 2005). For untargeted approaches like in the present thesis, the trapping material should have a low selectivity. Polydimethylsiloxane (PDMS) is a commonly used trapping material, for example in solid-phase microextraction (SPME) fibers or the Twister® system (GERSTEL GmbH & Co.KG, Mülheim an der Ruhr, Germany). PDMS is non-polar and traps volatiles by sorption rather than adsorption. Sorption is a relatively weak interaction (Kallenbach et al., 2014), which is why the sample recovery needs relatively little stringent methods minimizing analyte degradation (van Pinxteren et al., 2010). An alternative to commercially available products aimed specifically for volatile trapping, are standard PDMS tubes for laboratory use. These PDMS tubes are extremely cheap, robust, easy to handle, and lightweight compared to competing products. These qualities make them attractive for use under harsh fieldwork conditions (van Pinxteren et al., 2010), which is why we used them for volatile trapping in **Article II**. In contrast, the volatile trapping in **Article III** took place under lab conditions and was performed using steel traps filled with Tenax® TA (Markes International, Llantrisant, United Kingdom), a carbon-based sorbent that traps a wide range of compounds (C_6–C_{30}), making it suitable for untargeted volatilomics likewise. Trapping volatiles with Tenax® TA may be more expensive, and the equipment is heavier than PDMS tubes, but it results in fewer contaminations of the samples and the GC system.

After trapping, the second step is the release of the volatiles from the trapping material. In this thesis, it was done by thermodesorption. An inert gas (mobile phase) transports the volatiles to the subsequent cold trapping step, before transfer to the GC column. We used a DB-5ms ultra inert column (Agilent Technologies, Inc. Santa Clara, CA, USA), which is a fused silica column with a polyphenylmethylsiloxane film. Different interactions between the volatiles and the column material (stationary phase) led to the separation of the volatile blend. The time needed for a volatile to run through the column is called its “retention time” and is used to calculate the system-independent retention index, which is crucrial for later library search.

In the third step, the separated volatiles get transferred to the mass spectrometer. For this, we used a hybrid approach consisting of a quadrupole and a time-of-flight (Q-TOF) mass analyzer. The volatiles are selected based on their mass-to-charge ratio (*m/z*) by the quadrupole, fragmented in a collision cell, and analyzed in the TOF mass analyzer. The

fragmented volatiles are accelerated and need to travel a certain distance to the detector. Lighter fragments are faster, leading to volatile-specific mass spectra.

In the fourth and final step, the mass spectra are processed and statistically significant peaks are calculated for the library search which consists of retention indices comparison and mass spectra comparison. At this stage the volatiles are often referred to as “tentatively identified”, because their identification is completely based on library comparisons. To verify their presence in a sample, pure standard compounds need to be analyzed in the same GC/Q-TOF system and the resulting retention indices and mass spectra have to be compared.

WHAT MAKES THE LIFESTYLE OF SOCIAL SPIDERS SO SPECIAL?

The lifestyle of social insects like ants, termites, and bees is characterized, among others, by living in colonies and division of labor (Beshers and Fewell, 2001). They are considered to be highly successful because they are able to inhabit extreme environments, use diverse resources, and often outcompete other arthropods, dominating the insect biomass in many ecosystems (Wilson, 1987; Hölldobler and Wilson, 1990; Fisher et al., 2019). Sociality has likewise evolved within the class of arachnids, in 25 spider species to be precise (Lubin and Bilde, 2007). *Stegodyphus dumicola* is a social spider species living in large colonies of up to a few hundred individuals in southern and central Africa (Aviles, 1997; Lubin and Bilde, 2007). The spiders build nests composed of spider silk, leaves, branches, and prey carcasses, surrounded by three-dimensional catching webs that can span over meters (**Figure 4**) (own observation; Lubin and Bilde, 2007; Majer et al., 2018). The nests, which are often found in *Acacia* trees, protect the spiders from predators, heat, dehydration, and UV radiation and are the place where reproduction takes place (Seibt and Wickler, 1990; Henschel, 1998; Lubin and Bilde, 2007). While the females dominate the colonies (85 %) and perform colony activities like hunting, feeding, nest building, and brood care, the males do not seem to perform relevant colony activities (Henschel, 1998; Lubin and Bilde, 2007). At least they contribute to reproduction. A newly established nest is formed by a single female spider after mating, resulting in a genetically identical nest and in a strictly inbreeding mating system. Together with frequent extinction events, this results in extremely low genetic diversity (Lubin and Bilde, 2007; Settepani et al., 2014, 2016, 2017).

Figure 4 | Photograph of a *Stegodyphus dumicola* nest of approximately 20 cm. This is Figure S1A of **Arcticle II**.

This lifestyle, including high population densities and low genetic diversities, is likely to cause an increased risk for the spread of pathogens within populations (Schmid-Hempel, 1998; Wilson-Rich et al., 2009). This led us to hypothesize that antimicrobial compounds may play an important role in the pathogen defense of *S. dumicola*. Due to the complex nest structure of tightly woven silk structures with multiple narrow tunnels and chambers, soluble antimicrobial compounds are presumably very hard to spread within the nest, whereas volatiles can diffuse through the air, potentially reaching the whole nest. Indeed, antimicrobial volatiles were reported in ecosystems of social insect that are potentially involved in pathogen

protection (Chen et al., 1998; Wang et al., 2015) but there is no such information about social spider ecosystems yet, which is why we investigated that (**Articles II and III**).

ARE THERE ANTIMICROBIAL VOLATILES PRESENT IN SPIDER NESTS? (ARTICLE II)

It is very promising to search for antimicrobial volatiles in social spider ecosystems, because of the expected increased risk of infection and the complex structure of the spiders' nests, as discussed in the previous chapter. That is why we hypothesized the presence of antimicrobial volatiles in the ecosystem of *Stegodyphus dumicola* in **Article II**, which I will summarize in this chapter. We trapped *in situ* volatile samples from the nests, catching webs, and spider bodies at three different sampling sites in a north-south gradient in Namibia and analyzed them subsequently using GC/Q-TOF.

We found volatiles ranging from C_4 up to C_{24}. Whereas most of them were pure hydrocarbons or contained oxygen, some contained nitrogen, sulfur, or halogens. From the 53 tentatively identified volatiles, 21 (40 %) were described in literature as having antimicrobial activity, at least in mixtures like essential oils (**Table 1**). Furthermore, we tested the antimicrobial activity of five selected volatiles, namely acetophenone, 1-tetradecene, 1-decanal, 2-decanone, and 1,3-benzothiazole, against the putative spider pathogen *Bacillus thuringiensis* and the three human pathogens *Staphylococcus aureus*, *Escherichia coli*, and

Table 1 | List of identified compounds in the nest (N), web (W), and spider (S) volatilomes of the different sampling sites Otavi, Windhoek, and Stampriet with known antimicrobial activity. The antimicrobial activity of the volatiles was shown against bacteria (b) and/or fungi (f). The volatiles originated from plants (p), insects (i), algae (a), bacteria (b), and/or fungi (f). Antimicrobial activities and origins are based on literature data. Detection of volatiles in the samples is indicated by "×". This is Table 1 from **Article II**.

					Otavi			Windhoek			Stampriet		
Compound	**Class**	**Molecular Formular**	**Antimicrobial Activity**	**Compound Origin**	**N**	**W**	**S**	**N**	**W**	**S**	**N**	**W**	**S**
1-Heptanal	Aldehyde	$C_7H_{14}O$	b, f	p						×			
2-Ethylhexanol	Alcohol	$C_8H_{18}O$	b, f	p, f				×	×		×	×	
Acetophenone	Ketone	C_8H_8O	b	?	×			×					
1-Non-anal	Aldehyde	$C_9H_{18}O$	b, f	p	×						×		
Levomenthol	Alcohol	$C_{10}H_{20}O$	b, f	p		×							
2-Decanone	Ketone	$C_{10}H_{20}O$	b, f	p, b				×			×		
Dodecane	Alkane	$C_{12}H_{26}$	b, f	p, b							×		
1-Decanal	Aldehyde	$C_{10}H_{20}O$	b, f	p	×	×							
1,3-Benzothiazole	Benzothiazole	C_7H_5NS	b, f	p	×	×		×					
1-Undecanol	Alcohol	$C_{11}H_{24}O$	b	?								×	
1-Dodecene	Alkene	$C_{12}H_{24}$	b	p							×		
1-Tridecene	Alkene	$C_{13}H_{26}$	b	p, a	×	×		×	×		×		
2-Ethyl-3-hydroxyhexyl 2-methylpropanoate	Carboxylic Acid	$C_{12}H_{24}O_3$	b, f	p	×								
1-Tetradecene	Alkene	$C_{14}H_{28}$	b, f	p, f			×						
1-Dodecanal	Aldehyde	$C_{12}H_{24}O$	b, f	p							×		
Nerylacetone	Ketone	$C_{13}H_{22}O$	b	p	×	×						×	
1-Dodecanol	Alcohol	$C_{12}H_{26}O$	b	p		×	×	×			×		
Pentadecane	Alkane	$C_{15}H_{32}$	b, f	a, i							×	×	
Myristic acid	Carboxylic Acid	$C_{14}H_{28}O_2$	b, f	p	×						×		
Heneicosane	Alkane	$C_{21}H_{44}$	b	p							×	×	
Docosane	Alkane	$C_{22}H_{46}$	b, f	p							×	×	

Candida albicans. Most test organisms were sensitive to all volatiles. Thus, we could support our hypothesis by showing numerous volatiles that potentially protect the spiders from pathogen infections.

The origins of the volatiles can be diverse: the spiders themselves, microorganisms, the spiders' prey, the plants in which the nests are located, or other animals (including predators). Most of the tentatively identified volatiles have been reported in essential oils of plants, but some have been shown to be produced by microorganisms or insects (references in Table S2 of **Article II**). Interestingly, some volatiles we tentatively identified in *S. dumicola's* ecosystem have even been found in other social arthropod systems. For example, the antimicrobial volatile pentadecane was reported in the head and gaster of Argentine ants (Cavill and Houghton, 1974). Therefore, it seems plausible that the pentadecane we tentatively identified in the *S. dumicola* ecosystem originated from dead prey or hostile ants (**Figure 5**). However, one of the most likely origin of the volatiles is the microbiome of *S. dumicola,* because the microbiome of other arthropods is known to produce both soluble (Currie et al., 1999; Chouvenc et al., 2013; Mendes et al., 2013) and volatile (Musa Bandowe et al., 2009) antimicrobial compounds. Interestingly, a study investigating the nest microbiome of *S. dumicola* reported significant differences between sampling sites (Nazipi et al., 2021). Another study investigating the spider microbiome of *S. dumicola* even reported differences between nests from the same sampling site (Busck et al., 2020). The volatilome comparison between the three sampling sites likewise revealed clear differences. This suggests that the volatilomes might be (partly) shaped by the microbiome of *S. dumicola*. The microbiomes themselves can be shaped by factors like temperatures, humidity, prey, soil, and plant species

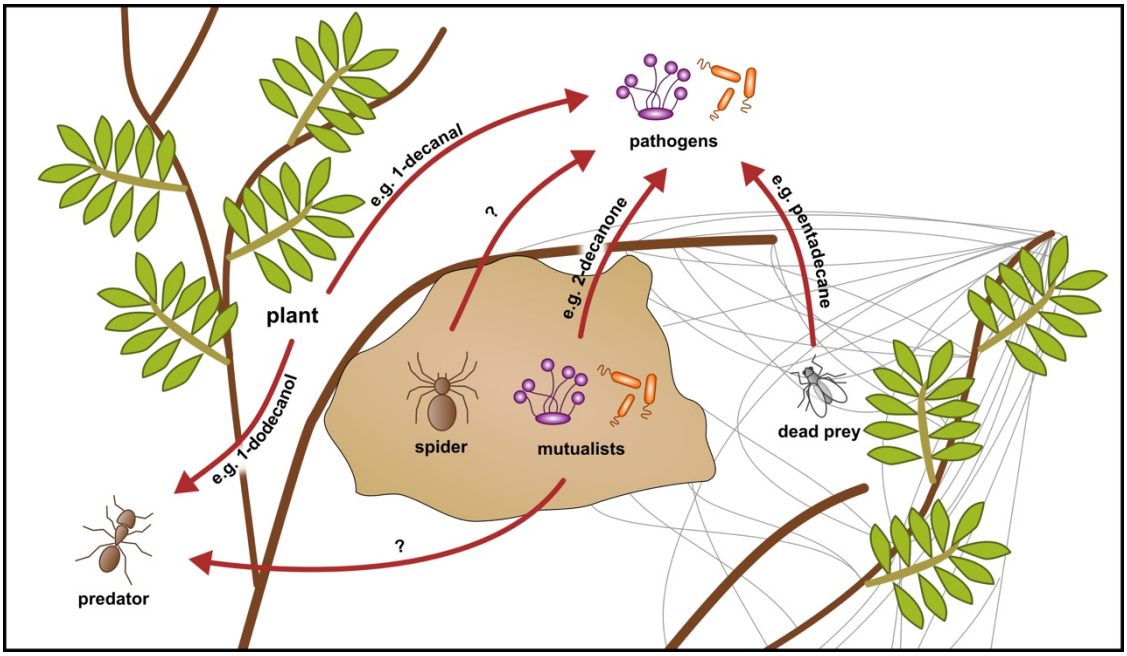


Figure 5 | Conceptual scheme of hypothetical volatile interactions in a social spider nest system. Volatiles can be originated from the spiders themselves, their microbiome, dead prey, or the plants and inhibit pathogens and predators (red arrows). The example volatiles were found in the analyzed volatilomes of the present study. Their origin and influence are assumed based on literature. This is Figure 5 from **Article II**.

(Reese and Dunn, 2018). This is supported by our findings of different temperatures, humidity levels, and plant species at the different sampling sites.

Notwithstanding the differences between the volatilomes some volatiles were found ubiquitously, suggesting a site-independent “core volatilome”. For example, 1-tridecene was found in all nest and web volatilomes and 1-dodecanol in all nest or web volatilomes. Both compounds are known to have antibacterial activities (Togashi et al., 2007; Kumar et al., 2011; Vairappan et al., 2012; Satmi and Hossain, 2016) and 1-dodecanol is even known to have insecticidal properties (Tabanca et al., 2014) making it conceivable as a protection for *S. dumicola* against hostile insects and bacterial pathogens alike (**Figure 5**). However, the origins of the volatiles remain speculative so far but there is still **Article III**.

DO SOCIAL SPIDER NEST MEMBERS PRODUCE ANTIMICROBIAL VOLATILES? (ARTICLE III)

Social spiders, like other social arthropods, are probably protected from pathogens by antimicrobial compounds. It is likely that these antimicrobial compounds include volatiles produced by microorganisms. In the final chapter of this introduction, I will summarize the main findings of **Article III,** the goal of which was to investigate whether the spider nest microbiome produces antimicrobial volatiles.

We analyzed the volatilomes of five microbial nest members *of S. dumicola* that were isolated and provided by our cooperation partners from Aarhus University (Nazipi et al., 2021, submitted). Four of the isolates were members of the core nest microbiome, namely *Massilia* sp. IC2-278, *Massilia* sp. IC2-477, *Sphingomonas* sp. IC-11, and *Aureobasidium* sp. CE_32. Their genera were found in all investigated spider nests and made up to approximately 65 % of the microbiome within a nest. We also investigated the volatilome of *Streptomyces* sp. IC-207, which does not belong to the core members, but *Streptomyces* species are generally known for their antimicrobial potential (Watve et al., 2001; Avalos et al., 2019).

Initially, we tested the antimicrobial activity of these selected nest microbiome members against two putative spider pathogens (Keiser et al., 2016; Shrestha et al., 2019), namely *Bacillus thuringiensis* and *Purpureocillium lilacinum.* The indirect two-chamber Petri dish approach revealed that most nest microbiome members released a volatile blend that inhibited at least one of the two putative spider pathogens (**Figure 6**). For example, both *Massilia* species significantly inhibited the growth of *B. thuringiensis*. In line with this finding, a recent study showed the antimicrobial potential of *Massilia* species via soluble compounds (Dahal et al., 2021) and another study identified numerous biosynthetic gene clusters suggesting *Massilia* species as potential producers of antimicrobial compounds (Miess et al., 2020). These results clearly show the antimicrobial potential of *S. dumicola* nest member's volatilomes.

Subsequently, we trapped the volatilomes of the (core) nest members, analyzed them using GC/Q-TOF, and detected 78 volatiles, 32 of which could be tentatively identified. Interestingly, our results revealed that producing more volatiles does not come with higher bioactivity. For example, while *Massilia* sp. IC2-477 produced three times more volatiles than *Massilia* sp. IC2-278 (**Figure 7**), both inhibited the bacterial putative pathogen but not the fungal one (**Figure 6**). Furthermore, *Aureobasidium* sp. CE_32 released the fewest volatiles from all investigated strains but was the only one that reduced the growth of both the bacterial and fungal putative pathogens (**Figures 7 and 8**). The presence of five volatiles was confirmed, namely crotyl alcohol, dimethyl disulfide, 2,5-dimethylpyrazine, dimethyl trisulfide, and benzonitrile. Subsequent antimicrobial tests revealed all five volatiles had antimicrobial or antifungal properties, or even both, in the case of crotyl alcohol and dimethyl trisulfide.

Interestingly, some (tentatively) identified volatiles produced by the invested nest members are known in other (social arthropod) ecosystems. For example, *Streptomyces* sp. IC-207 produced isoprenol which has also been found in propolis ("bee glue"), a substance known for its antimicrobial activities (Kaskoniene et al., 2014). Another study reports isoprenol to be produced by *Pseudomonas yamanorum,* which protects bats from the white-nose syndrome (Li et al., 2021). Termite queen pheromones contain 2-methyl-1-butanol, a compound we tentatively identified in the volatilome of *Aureobasidium* sp. CE_32, and which was suggested as a protectant for termite eggs (Matsuura and Matsunaga, 2015).

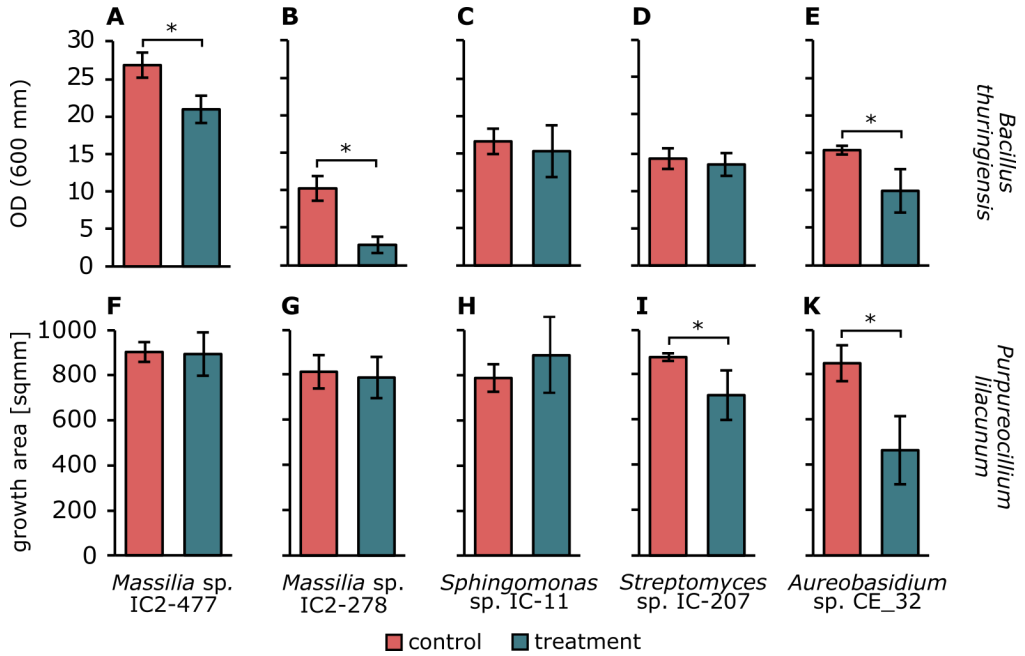


Figure 6 | Antimicrobial activity of the volatilomes of the nest microbiome members *Massilia* sp. IC2-477, *Massilia* sp. IC2-278, *Sphingomonas* sp. IC-11, *Streptomyces* sp. IC-207, and *Aureobasidium* sp. CE_32 against the putative spider pathogens *Bacillus thuringiensis* (A-E) and *Purpureocillium lilacinum* (F-K). The error bars show the standard deviations. The * indicate significant differences ($p \leq 0.05$); *t*-test; $n = 6$; OD = optical density. This is Figure 1 from **Article III**.

The comparison of the *in vitro* volatilomes from **Article III** with the *in situ* volatilomes from **Article II** revealed six volatiles with the same retention indices, although it should be noted that a comparison based on only the retention indices would be highly speculative. Furthermore, we tentatively identified two dimethylundecanes produced by *Sphingomonas* sp. IC-11 in **Article III** and two other dimethylundecanes in **Article II**, but these alkanes are not known for any bioactivity yet. We did find clear indications that (core) nest members of *S. dumicola* contribute to the antimicrobial activity of the nest volatilome, and therefore potentially to pathogen protection. However, the volatilomes inside spiders' nests are probably more complex than the volatilomes we analyzed, because they are most likely a combination of volatilomes of bacteria, fungi, surrounding plants, the spiders themselves, prey, and other organisms (**Article II**).

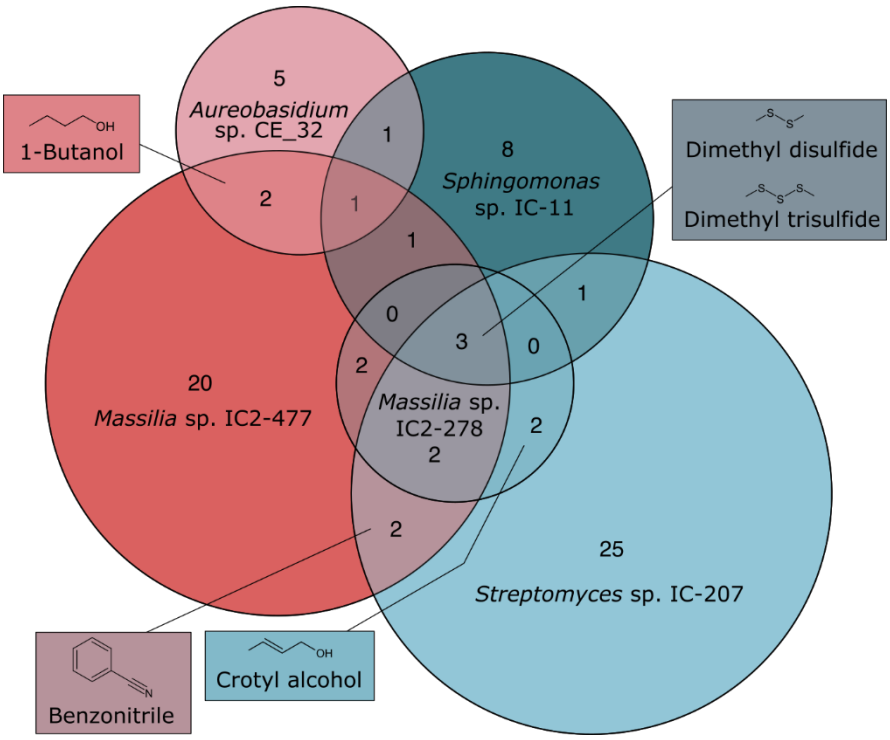


Figure 7 | Volatilome comparison of the nest microbiome members *Massilia* sp. IC2-477, *Massilia* sp. IC2-278, *Sphingomonas* sp. IC-11, *Streptomyces* sp. IC-207, and *Aureobasidium* sp. CE_32 by an Euler plot. The plot is based on all identified and unknown volatiles. The areas are proportional to the number of volatiles. The boxes show those volatiles that were produced by several members and could be identified. Notice that Euler plots only describe data by approximation. This is Figure 2 from **Article III**.

OUTCOME AND FUTURE PERSPECTIVES

This thesis reviewed the potential of antimicrobial volatiles (**Article I**), proved the presences of numerous antimicrobial volatiles in the ecosystems of social spiders (**Article II**), and finally revealed microbial spider nest members as sources of antimicrobial volatiles (**Article III**). This results in two main achievements:

First, the presence of antimicrobial volatiles in the ecosystem of *S. dumicola* helps explain the spider's ecological success under challenging conditions, including a high colony density and low genetic diversity. For now, we lack information about *in situ* concentrations of the volatiles, but the high number of antimicrobial volatiles in the volatilomes and the high antimicrobial potential of pure volatiles suggest a role in pathogen defense. Untargeted volatilomics (as done in this thesis) cannot be combined with quantification of volatile concentrations. In future studies, selected antimicrobial volatiles could be analyzed in both *in situ* and *in vitro* experiments, making it possible to quantify their concentrations. Even though the general concentrations of volatiles in spider nests may be rather low, they might accumulate in enclosed spaces (micro incubators) and reach concentrations high enough to act as antimicrobials, like discussed in soil environments (Rillig et al., 2017).

Second, we established that the volatilome of *S. dumicola* is a highly promising and novel source of antimicrobial volatiles that may ultimately help to counter the antimicrobial resistance crisis. It is worth noting that the antimicrobial volatiles discussed in this thesis (**Articles II** and **III**) were identified based on database comparisons. Approximately 60-75 % of the total number of volatiles could not be identified, leaving a high potential to find further volatiles with antimicrobial activity. In the future, antimicrobial volatiles could potentially replace traditional antibiotics or be used in combination with those to increase the antimicrobial activity (**Article I** and **III**). The physicochemical properties of volatiles seem to be impractical for pharmaceutical treatments. In fact, numerous volatiles are liquid if not solid at room temperature. Furthermore, volatiles could be inhaled, for example, to treat lung infections (Avalos et al., 2019). In addition, to being used to treat pathogen infections, volatiles could be used to detect pathogen infections, as shown in infected swine (Traxler et al., 2018) and humans (Neerincx et al., 2016).

For a long time, (antimicrobial) volatiles got little attention from scientists. But now that innovative approaches are needed to counter the antimicrobial resistance crisis, it is time to try and develop volatile-based treatments against pathogens. Since volatiles are ubiquitously produced and come in a wide range of chemical classes, they represent a highly potent group of compounds to counter the antimicrobial resistance crisis.

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AUTHOR CONTRUBUTIONS

ARTICLE I

Alexander Lammers and Paolina Garbeva conceptualized and wrote the manuscript. Alexander Lammers, Michael Lalk, and Paolina Garbeva reviewed and edited the manuscript. Alexander Lammers illustrated the figures. Michael Lalk and Paolina Garbeva made the funding acquisition and supervised the project.

ARTICLE II

Alexander Lammers, Andreas Schramm, Hans Zweers, Michael Lalk, Paolina Garbeva, and Trine Bilde designed the study. Alexander Lammers performed the volatilome trapping, GC/Q-TOF data analysis, antimicrobial testing, and wrote the manuscript with support from Andreas Schramm, Paolina Garbeva, and Trine Bilde. Tobias Sandfeld assisted in planning and realization of the fieldwork. Hans Zweers performed the GC/Q-TOF analysis.

ARTICLE III

Alexander Lammers, Seven Nazipi, Trine Bilde, Andreas Schramm, Paolina Garbeva, and Michael Lalk designed the study. Alexander Lammers performed the volatilome trapping, GC/Q-TOF data analysis, antimicrobial testing, and wrote the manuscript with support from Paolina Garbeva, Michael Lalk, Andreas Schramm, and Trine Bilde. Seven Nazipi isolated the microbial strains. Hans Zweers performed the GC/Q-TOF analysis.

Prof. Dr. Michael Lalk

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ARTICLE I

AIR AMBULANCE:
ANTIMICROBIAL POWER OF BACTERIAL VOLATILES

Alexander Lammers, Michael Lalk and Paolina Garbeva

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Review

Air Ambulance: Antimicrobial Power of Bacterial Volatiles

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Abstract: We are currently facing an antimicrobial resistance crisis, which means that a lot of bacterial pathogens have developed resistance to common antibiotics. Hence, novel and innovative solutions are urgently needed to combat resistant human pathogens. A new source of antimicrobial compounds could be bacterial volatiles. Volatiles are ubiquitous produced, chemically divers and playing essential roles in intra- and interspecies interactions like communication and antimicrobial defense. In the last years, an increasing number of studies showed bioactivities of bacterial volatiles, including antibacterial, antifungal and anti-oomycete activities, indicating bacterial volatiles as an exciting source for novel antimicrobial compounds. In this review we introduce the chemical diversity of bacterial volatiles, their antimicrobial activities and methods for testing this activity. Concluding, we discuss the possibility of using antimicrobial volatiles to antagonize the antimicrobial resistance crisis.

Keywords: volatile organic compounds; volatiles; chemical ecology; metabolomics; antibacterial; antifungal; antibiotics; antimicrobial resistance crisis

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1. Antimicrobial Resistance: A Global Crisis

Many would argue that the discovery of antibiotics changed the world of medicine. Penicillin is often reported as the first antibiotic available to the public, whereas it was actually sulfamidochrysoidine [1,2]. In contrast to penicillin, sulfamidochrysoidine, which was sold under the trade name Prontosil, was toxic for humans and disappeared quickly from the market and history books [1]. However, with the introduction of penicillin in the 1940s, antibiotics have saved millions of lives and the subsequent years are often referred as the "golden age" of antibiotics due to the discovery of numerous novel classes [3]. In fact, in the end of the 1960s around 24 novel classes of antibiotics were introduced to the market [2], but since the 1970s only three classes, namely pseudomonic acids [4,5], oxazolidiones [6] and lipopeptides [7] have been introduced to the market. One of the latest promising antibiotics is the peptide teixobactin, which due to its highly conserved targets is unlikely to induce resistance but is still not available on the market [8]. Interestingly, Alexander Fleming understood the fragility of the powerful tool antibiotic and warned at his Nobel Prize speech in 1945: "The time may come when penicillin can be bought by anyone in the shops. Then, there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant" [9]. In his speech, Fleming indicated the risk of antimicrobial resistance—an issue that we are facing today.

How is it possible that, despite Alexander Fleming's warning, resistant pathogens could become a global issue? In human medicine antibiotics are often overused as well as misused. That means that antibiotics are prescribed as a prophylactic or the actual pathogen is not identified before prescription. Furthermore, patients may take antibiotics without referring to a doctor. Additionally, the massive and prophylactic use of antibiotics

in agriculture causes resistance which may be transferred to humans. Globalization makes the spread of resistant pathogens very easy and the hurdles for (economically driven) pharmaceutical companies are very high. However, these are only the main reasons and have already been reviewed in detail [10]. This misuse of antibiotics has led to the development of numerous antibiotic-resistant pathogens resulting in an antimicrobial resistance crisis on a global scale [10–12]. There are already at least 700,000 deaths caused every year by drug-resistant pathogens globally [13] and scientists predict that by 2050 antibiotic resistance could be responsible for over 10,000,000 deaths per year [14]. Moreover, the global COVID-19 pandemic has caused an increased use of antibiotics due to bacterial co-infections or prophylactic treatment with antibiotics to avoid those co-infections [15]. In order to address this crisis, we need to explore alternatives to classical antibiotics. Numerous pharmaceutical options to counter the antimicrobial resistance crisis are under discussion [16,17]. One approach is to discover novel sources for antimicrobial compounds that can be developed into future treatments. In recent years, more and more studies have reported volatiles with antimicrobial activity which indicates that volatiles might play an important role in countering the antimicrobial resistance crisis [18–21]. For example, the new volatile antibiotics albaflavenone and pentalenolactone produced by *Streptomyces coelicolor* and *Streptomyces avermitilis*, respectively were discovered [22].

In this review we introduce the chemical characteristics of volatiles, provide an overview of antimicrobial volatiles of bacterial origin and the main methods used for testing their antimicrobial activity. Concluding, we discuss the potential role of volatiles as a novel class of antimicrobials.

2. Biochemistry of Volatiles: Diverse and Diffusive

Volatiles form a chemical class of molecules that all have one characteristic in common: their high vapor pressure at ambient temperatures [23]. Additionally, volatiles are characterized by their low molecular weights of a maximum of 200–500 Dalton, low boiling points and often lipophilic moieties [23–25]. Volatiles significantly differ from soluble compounds in one key characteristic: they do not depend on solvents. Although many volatiles are non-polar showing low solubility in water due to their restricted number of functional groups, this solubility is sufficient to allow dissemination into the water phase. Hence, volatiles spread fast in both the gas and water phase [26]. Via the gaseous phase, volatiles can spread in highly complex ecosystems such as soil [27], insect nests [28] and spider nests [29] and can fulfill functions such as communication [30] and antimicrobial defense [31] which cannot be performed by solvents due to the lack of effective spreading.

Volatiles belong to diverse chemical classes such as hydrocarbons, aromates, alcohols, aldehydes, acids, esters, amines and thiols [23]. Bacterial volatiles in particular were for the first time systemically reviewed by Schulz and Dickschat in 2007 describing in detail the biosynthesis of common volatiles like fatty acids or sulfur-compounds which are produced by most bacteria but also rare volatiles like halogenated compounds [32]. Volatile biosynthesis is usually based on pyruvate and therefore takes place in primary metabolism (Figure 1) [24,33]. Pyruvate can be directly metabolized to short acids or alcohols [33]. *Bacillus* spp. for example, which are well known for producing volatiles as we will discuss later in this review, produces mainly 2,3-butanediol and acetoin via fermentation [34].

Under aerobic conditions pyruvate will be metabolized to acetyl-CoA and can enter the fatty acid anabolism, citric acid cycle or be converted to terpenes. The fatty acid metabolism results mainly in alkanes, alkenes, aliphatic alcohols and ketones. The β-oxidation with acetyl-CoA results only in fatty acids with even numbers, for odd-chain fatty acids propionyl-CoA replaces one acetyl-CoA in the final step [33]. Typical volatile products from the fatty acid pathway are aldehydes such as nonanal, ketones such as nonan-2-one or fatty acids such as nonanoic acid [26].

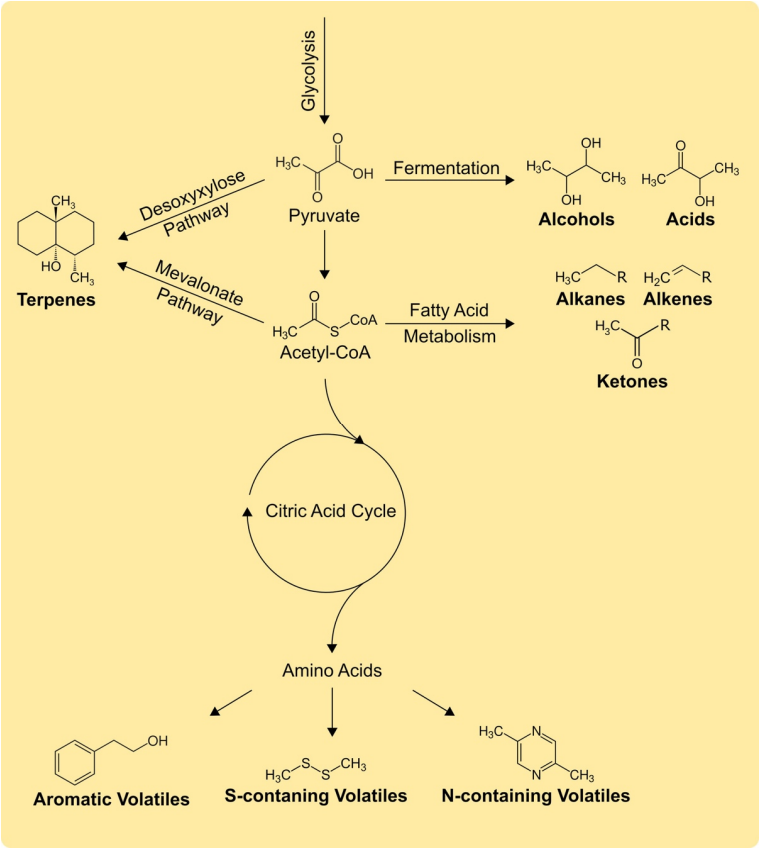

Figure 1. Overview of main biochemical pathways for the production of bacterial volatiles. The chemical structures show representative examples: alcohols (2,3-butanediol), acids (acetoin), alkanes (general structure), alkenes (general structure), ketones (general structure), terpenes (geosmin), aromatic volatiles (2-phenylethanol), S-containing volatiles (dimethyl disulfide) and N-containing volatiles (2,5-dimethylpyrazine). Details are described in the main text.

When acetyl-CoA enters the citric acid cycle it is metabolized to the precursors of most amino acids, which act again as precursors for aromatic, nitrogen-containing and sulfur-containing volatiles [24]. Aromatic volatiles are metabolized based on aromatic amino acids or directly via the shikimate pathway. 2-Phenylethanol for example, a common aromatic volatile compound, can be metabolized based on the amino acid phenylalanine [32,33]. Pyrazines are usually based on amino acids due to their nitrogen-containing aromatic ring. Additionally, sulfur-containing volatiles such as dimethyl disulfide and dimethyl trisulfide which are produced by most bacteria are based on methionine [32].

Another important group of volatiles are terpenes, which are well known to be present in essential oils [35] but in recent years have also been discovered frequently in bacterial volatile blends [36]. Terpenes are synthesized via the mevalonate or desoxyxylulose pathway [26]. The mevalonate pathway starts with acetyl-CoA from the glycolysis and was for a long time assumed to be the only way to biosynthesize isopentenyl diphosphate and dimethylallyl diphosphate, the precursors of terpenes. However, the desoxyxylulose

pathway starts with pyruvate, the precursor of acetyl-CoA [37,38]. The sesquiterpene geosmin, which is produced by actinomycetes, myxobacteria and cyanobacteria has a characteristic soil-like smell [32,33]. Interestingly, different geosmin synthases were found in actinobacteria compared to myxobacteria and cyanobacteria [36].

Alongside organic volatiles, bacteria produce inorganic volatiles such as hydrogen sulfide, hydrogen cyanide, nitric oxide or ammonia [36]. Ammonia for example is produced in high amounts by *Streptomyces* spp. and is produced within amino acid catabolism [39,40]. Moreover, ammonia was shown to be antimicrobial against Gram-positive and -negative bacteria and can act therefore as a long-distance (several centimeters) antibiotic [39].

3. Bacterial Antimicrobial Volatiles: An Overview

Recently, an increasing number of studies have revealed individual volatiles or volatile blends of bacterial origin with antimicrobial activities [18]. These studies indicate that the antimicrobial potential of volatiles is as diverse as their biochemistry (Table 1). A bulk of the investigated volatiles were reported for their antifungal activities and cause for reduced hyphal extension and/or hyphal biomass as well as spore germination. For example, the volatile blends produced by *Paenibacillus polymyxa* Sb3-1 and *Bacillus velezensis* I3 were shown for their antifungal activities [41,42]. However, several studies also reported bacteria that produce antibacterial [43] or anti-oomycete volatiles [44]. Beyond that, some volatiles were reported for their broad antimicrobial spectrum. For example, the volatile 2,5-*bis*(1-methylethyl)-pyrazine produced by *Paenibacillus* sp. AD87 revealed a broad-spectrum activity against a range of human and plant pathogens. The volatile inhibited the bacterial pathogens *Escherichia coli* and *Staphylococcus aureus*, the fungal pathogens *Fusarium culmorum* and *Rhizoctonia solani* as well as the yeast *Candida albicans* [21,45]. At the same time 2,5-*bis*(1-methylethyl)-pyrazine showed very low toxicity on mammalian cells [45]. Another example of volatiles with broad spectrum antimicrobial activity is γ-Butyrolactones, active against fungi, yeasts, and bacteria [46].

Table 1. Overview of recent (2017–2021) studies showing the antimicrobial activity of bacterial volatiles. The studies are ordered alphabetically by the volatile producer's name. Only studies that trapped the antimicrobial volatiles in the gas phase and/or showed the antimicrobial effect via the gas phase are listed. blend = The volatile blend might be analyzed in the cited study but only the antimicrobial activity of the blend was tested. f = antifungal, b = antibacterial, o = anti-oomycete.

Volatile Producer	Volatile(s)	Bioactivity	Reference
Bacillus amyloliquefaciens CPA-8	blend 1,3-pentadiene thiophene acetoine	f	[47]
Bacillus amyloliquefaciens DA12	blend	f	[48]
Bacillus amyloliquefaciens FZB42	blend 1,2-benzisothiazol-3(2*H*)-one benzaldehyde other	b	[49]
Bacillus amyloliquefaciens L3 other	blend 2-heptanone 2-ethyl-1-hexanol 2-nonanone other	f	[50]
Bacillus artrophaeus LSSC22	blend 1,2-benzisothiazol-3(2*H*)-one other	b	[49]
Bacillus cereus CHP20	blend	o	[44]
Bacillus megaterium KU143	blend	f	[51]

Table 1. *Cont.*

Volatile Producer	Volatile(s)	Bioactivity	Reference
Bacillus pumilus TM-R	blend	f	[52]
Bacillus siamensis LZ88	blend	f	[53]
Bacillus (diverse spp.)	blend	f	[54]
Bacillus (diverse spp.)	blend 2-undecanone benzothiazole 1,3-butadiene *N*,*N*-dimethyldodecylamine other	f	[55]
Bacillus sp. BO53	blend	b	[43]
Bacillus sp. D13	blend	b	[56]
Bacillus sp. TM-I-3	blend	f	[57]
Bacillus subtilis CHP14	blend	o	[44]
Bacillus subtilis FA26	blend benzaldehyde nonanal benzothiazole acetophenone	b	[58]
Bacillus subtilis M29	blend 1-butanol acetic acid butyl ester 1-heptylene-4-alcohol 3-methyl-3-hexanol other	f	[59]
Bacillus velezensis BUZ-14	blend diacetyl benzaldehyde isoamyl alcohol other	f	[41]
Bacillus velezensis G341	blend	f	[60]
Bacillus velezensis I3	blend	f	[41]
bacterial community	blend	f	[31]
Cronobacter muytjensii JZ38	blend	o	[61]
Frigoribacterium endophyticum CHP33	blend	o	[44]
Microbacterium testaceum KU313	blend	f	[51]
Paenibacillus sp. AD87	2,5-*bis*(1-methylethyl)-pyrazine	b, f	[21]
Paenibacillus polymyxa Sb3-1	blend	f	[42]
Proteus mirabilis 04	blend	b	[62]
Pseudoalteromonas sp. GA327	blend	b	[43]
Pseudomonas chlororaphis subsp. *aurantiaca* KNU17Pc1	blend	f	[63]
Pseudomonas chlororaphis subsp. *aureofaciens* SPS-41	blend 3-methyl-1-butanol phenylethyl alcohol 2-methyl-1-butanol other	f	[64]
Pseudomonas protegens AS15	blend	f	[51]

Table 1. *Cont.*

Volatile Producer	Volatile(s)	Bioactivity	Reference
Pseudomonas protegens CHA0	dimethyl trisulfide 2-ethylhexanol ammonium hydroxide phenol acetophenone 1,3-diphenylpropane 3-phenylpropiophenone	f	[65]
Pseudomonas putida BP25	blend 2-ethyl-5-methylpyrazine	f	[66]
Pseudomonas putida BP25	2,5-dimethyl pyrazine 2-methyl pyrazine 2-ethyl-5-methyl pyrazine 2-ethyl-3,6-dimethyl pyrazine dimethyl trisulfide	b, f, o	[67]
Pseudomonas stutzeri E25	blend dimethyl disulfide	f	[68]
Sphingobacterium multivorum Bel3-4	blend	f	[54]
Stenotrophomonas maltophilia CR71	blend dimethyl disulfide	f	[68]
Stenotrophomonas maltophilia (TD1 and GH1-5)	blend	f	[54]
Streptomyces alboflavus TD-1	blend anisole dimethyl trisulfide β-pinene benzenamine 1,5-cyclooctadiene	f	[69]
Streptomyces fimicarius BWL-H1	phenylethyl alcohol ethyl phenylacetate methyl anthranilate α-copaene caryophyllene methyl salicylate 4-ethylphenol	o	[70]
Streptomyces lavendulae SPS-33	blend 2-methyl-1-butanol 3-methyl-1-butanol pyridine phenylethyl alcohol other	f	[71]
Streptomyces sp. MBT11	blend	b	[39]
Streptomyces venezuelae (ATCC 15439)	blend	b	[39]
Streptomyces yanglinensis 3–10	blend methyl 2-methylbutyrate 2-phenylethanol β-caryophyllene	f, o f f f	[72]
Xenorhabdus szentirmaii PAM 25	blend	f	[73]

3.1. Sulfur-Containing Volatiles

Often, described antimicrobial volatiles of bacterial origin are alcohols, pyrazines and sulfides (Table 1). Especially sulfur-containing volatiles such as dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide are often reported because they are commonly produced

by bacteria and apparently have strong antimicrobial activities [26]. For example, dimethyl disulfide has antibacterial potential, as it revealed bacteriostatic effects against the two plant pathogens *Agrobacterium tumefaciens* and *Agrobacterium vitis* [74]. However, the volatile is also known for its antifungal activity. Currently, dimethyl disulfide is used as a novel fumigant (PALADIN®) to target soil-borne plant pathogens [25]. The chemically related volatile dimethyl trisulfide significantly inhibited the growth of three human pathogens *Serratia marcescens*, *Escherichia coli* and *Staphylococcus aureus* [75]. As well as linear sulfur-containing compounds, scientists also report from aromatic sulfur-containing compounds. Gotor-Vila reported thiophene in the volatilome of *Bacillus amyloliquefaciens* CPA-8 and showed its antifungal activity [47].

3.2. Bacillus and Streptomyces as Volatile-Producers

Most likely, all bacterial genera produce volatiles, but *Bacillus* species especially are often reported to produce volatiles with antimicrobial potential (Table 1). For example, volatiles emitted by *Bacillus amyloliquefaciens* FZB42 including benzaldehyde, 1,2-benzisothiazol-3(2*H*)-one and 1,3-butadiene showed strong inhibitory activities against *Ralstonia solanacearum*, a bacterial plant pathogen causing wilt disease [49]. Other studies report the same from *Bacillus amyloliquefaciens* strains but with antifungal activities [47,48,50]. Alongside *Bacillus*, *Streptomyces* species were also often investigated because the genus is well known for its antimicrobial potential, including volatiles [39,76]. For example, terpenoid volatiles are abundantly emitted by *Streptomyces* species and pose interesting antimicrobial properties. The soil isolate, *Streptomyces albidoflavus*, was shown to produce a sesquiterpene, namely albaflavenone, with antibacterial properties [77]. Lately, albaflavenone was isolated from other *Streptomyces* species and fungi [78,79]. Another sesquiterpene compound with antibacterial activity is dihydro-β-agarofuran, produced by *Streptomyces* species [80]. The antimicrobial volatile pentalenolactone emitted by *Streptomyces roseogriseus* was discovered to possess antibacterial activity against Gram-positive and Gram-negative bacteria. Furthermore, anisole, emitted by *Streptomyces albulus*, was reported to inhibit the growth of fungal plant pathogens *Sclerotinia sclerotiorum* and *Fusarium oxysporum* [81].

3.3. Co-Cultivation and Volatile Blends

Some studies revealed that the co-cultivation of different microbial strains can influence the metabolism of bacteria (Table 2). For example, *Paenibacillus* sp. AD87 was shown to produce the antimicrobial volatile 2,5-*bis*(1-methylethyl)-pyrazine when cultivated alone. After co-cultivation of *Paenibacillus* sp. AD87 together with the phylogenetically different strain *Burkholderia* sp. AD24, the headspace concentration of 2,5-*bis*(1-methylethyl)-pyrazine was increased [21]. Due to the growth inhibiting effect of 2,5-*bis*(1-methylethyl)-pyrazine on *Burkholderia* sp. AD24, it is likely that the volatile production of *Paenibacillus* sp. AD87 was increased as a response to the competition. As well as the increased production of the pyrazine, the co-cultivation resulted in a changed gene expression in both bacteria. For example, *Burkholderia* sp. AD24 showed an increased expression of a type IV secretion system gene which is involved in virulence. *Paenibacillus* sp. AD87 showed increased expression of genes involved in antibiotic resistance. Another study cultivated five bacterial strains together, among others likewise *Paenibacillus* sp. AD87 and *Burkholderia* sp. AD24 [82]. The analysis of the collective volatile blend of the five bacteria revealed among others 2,5-*bis*(1-methylethyl)-pyrazine as well. Interestingly, the pyrazine was only found in the collective volatile blend but neither in the volatilome of *Paenibacillus* sp. AD87 nor in one of the others indicating production activation by the co-cultivation. Rybakova et al. co-cultivated the bacterium *Paenibacillus polymyxa* Sb3-1 with the fungus *Verticillium longisporum* EVL43 resulting in several up- and downregulations of the volatile blends of both strains, including volatiles that are most likely related to antimicrobial defense [42]. Furthermore, the corporate production of volatiles was shown under lab conditions [83]. By co-cultivation of *Serratia plymuthica* 4Rx13 and *Staphylococcus delphini* without physical contact they produced corporately the volatiles schleiferon A and B. Separately, none of

the bacteria was able to produce those products. Furthermore, Abis et al. analyzed the relation between microbial diversity and volatile emission in general [84]. Interestingly, they could show that a reduced microbial diversity in soil correlates with an increased volatile emission and a smaller number of released volatiles. They discussed that these findings might be caused by a bacterial volatile absorption. However, it is certain that the microbial diversity and community influence the volatile blend in an ecosystem, even when the detailed relations still remaining unknown.

Table 2. Examples of bacterial volatiles that were upregulated or downregulated in co-cultures. blend = The volatile blend might be analyzed in the cited study but only the antimicrobial activity of the blend was tested. f = antifungal, b = antibacterial, o = anti-oomycete, na = not analyzed.

Co-Culture	Volatile(s)	Bioactivity	Reference
Burkholderia sp. AD24 *Paenibacillus* sp. AD87	2,5-*bis*(1-methylethyl)-pyrazine	b, f	[21]
Burkholderia sp. AD024 *Paenibacillus* sp. AD087 *Dyella* sp. AD056 *Janthinobacterium* sp. AD080 *Pseudomonas* sp. AD021	2,5-*bis*(1-methylethyl)-pyrazine other	na	[82]
Chryseobacterium sp. AD48 *Tsukamurella* sp. AD106	blend dimethyl trisulfide other	b, f, o b	[75]
Janthinobacterium sp. AD80 *Dyella* sp. AD56	blend dimethyl trisulfide other	f, o b	[75]
Paenibacillus polymyxa Sb3-1 *Verticillium longisporum* EVL43	blend trans-2,2,4,5-tetramethyl-1,3-dioxolane 1-butanol other	f na na	[42]
Serratia plymuthica 4Rx13 *Staphylococcus delphini*	schleiferon A and B	na	[83]

Mixtures of volatiles may result in increased antimicrobial activities compared to single volatiles. For example, a mix of four monoterpenes (γ-terpinene, 1S-α-pinene, β-pinene and β-myrcene) revealed strong antibacterial activity against the pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus* [85]. However, as single compounds they revealed little or no antimicrobial activity. Furthermore, for a number of fungal and bacterial isolates, antimicrobial activities of their volatile blend are reported, but the compounds responsible for this activity remained unknown (Table 1). It is plausible that not a single volatile but a mix of compounds is responsible for this activity.

3.4. Modes of Action and Abiotic Factors

Although, for many microbial volatiles, powerful antimicrobial activities have been reported, little is known about modes of action of these molecules on the target organisms. Some microbial volatiles can interfere with well-known bacterial chemical communication systems like N-acylhomoserine lactones' (AHLs) quorum-sensing. Bacteria use AHLs' quorum sensing to regulate certain phenotype expressions, such as biofilm formation, virulence factor expression, motility and others. Many lactones (10-methylundec-2-en-4-olide, 10-methylundec-2-en-3-olide, 10-methyldodecan-4-olide, 10-methyldodecan-5-olide, others) positively or negatively influenced the quorum-sensing bacterial communication. This influence could be due to the structural similarity between lactones and AHLs. However, other classes of volatiles such as dimethyl disulfide could also impact bacterial quorum sensing communication by significantly suppressing the transcription of AHLs synthase genes [86]. The above discussed 2,5-*bis*(1-methylethyl)-pyrazine resulted in more direct

damages which depend interestingly on the concentration. At high levels, the volatile resulted in DNA damage, whereas at low levels cell-wall damages were observed [45]. Likewise, another study investigating the volatile blend activity of a *Streptomyces* species against the oomycete plant pathogen *Peronophythora litchi* showed, among others, cell-wall damages [70]. A study investigating the antimicrobial activity of the monoterpenes linalyl acetate, menthol and thymol indicates that those volatiles modify the membrane permeability which causes leakage of intracellular material [87]. Furthermore, it is likely that the altered membrane allows the volatiles to enter the cells and might cause further damages. Nevertheless, detailed information about the modes of actions of antimicrobial volatiles are lacking yet and need further investigation. Moreover, detailed information about volatile concentrations are mostly lacking which makes clear statements about the ecological relevance of often challenging volatiles [88].

Additionally, the emission of microbial volatiles is influenced by various abiotic factors such as nutrient availability, temperature and pH. For example, a nutrient-poor growing condition triggered higher levels of terpene emission at an early growth stage of the fungal isolate *Fusarium culmorum* [82]. It is plausible that such compounds have strong antimicrobial activity and are important for the producing organisms to survive under competitive interactions. Another study showed changes in antifungal activity of the volatile blend of *Bacillus amyloliquefaciens* CPA-8 when cultivated on different media [47]. Similarly, the production of antifungal volatiles by the mycophagous soil bacterium *Collimonas* was strongly influenced by different nutrient conditions [89].

4. Antimicrobial Activity of Volatiles: The Testing Methods

The approaches to test the antimicrobial activity of volatiles can be divided into two main categories: indirect and direct (Figure 2). In indirect approaches the volatiles need to diffuse through the gas phase (usually air) to influence the test organisms. In contrast, direct approaches allow the volatiles to make contact with the test organisms via the liquid phase (usually water) and do not need to diffuse through the gas phase.

One of the most common indirect approaches is the use of two-chamber Petri dishes (Figure 2A) [27,75]. Two-chamber Petri dishes contain a physical wall to divide the inside space into two chambers, making it possible to fill each side with a different media. One side can be inoculated with volatile producing organism or pure volatiles, whereas the other side is inoculated with test organism. After a common incubation, the growth of the test organism is analyzed, e.g., by counting colony forming units or analyzing the growth area. The Petri dishes are commercially available with two, three, or four chambers allowing combination of several organisms or pure compounds with each other. In contrast, the double plate approach is based on standard Petri dishes (Figure 2B) [30]. The bottom and top parts can be filled with different media, whereas one part is inoculated with the volatile producing organisms or pure volatiles and the other part with test organisms. The growth of the test organisms under exposure of the volatiles can be analyzed similarly to the two-chamber Petri dish approach. The vial approach is based on vials filled with solutions containing volatiles (Figure 2C) [90]. The lid contains filter paper with a defined inoculum of test organisms and is exposed to the volatiles before it is incubated in liquid medium to analyze the growth of the test organism. Recently, the AntiBio Vol approach was published (Figure 2D) [91]. Defined biofilms in a 24-well plate were placed upside-down on a second 24-well plate filled with solutions containing volatiles. After common incubation the biofilm is transferred to fresh, liquid broth, incubated shortly and the biomass is analyzed.

In contrast to indirect approaches, direct approaches do not compel the volatiles to actually diffuse through the gas phase, which comes with the advantage of better control of the volatile concentrations but the drawback that those approaches are not suitable with many volatiles due to their frequent lipophilic moiety, which may require the use of organic solvents [23]. The agar diffusion test is widely used to test the antimicrobial activity of pure compounds on solid media (Figure 2E) [46,92]. Cotton discs prepared with pure compounds are placed on agar plates that were inoculated with test organisms. The test

compounds diffuse through the agar and may result in a zone of inhibition (ZOI) around the cotton disc. The application of different concentrations of test compounds can be used to determine the minimal inhibitory concentration (MIC). However, a more common approach to investigate the MIC is the two-fold dilution approach which is based on liquid media (Figure 2F). For the approach a defined concentration of the test compound in liquid broth is prepared and subsequently several times 1:2 diluted [93]. Often, this approach is performed using 96-well plates to reach high throughputs with little material usage.

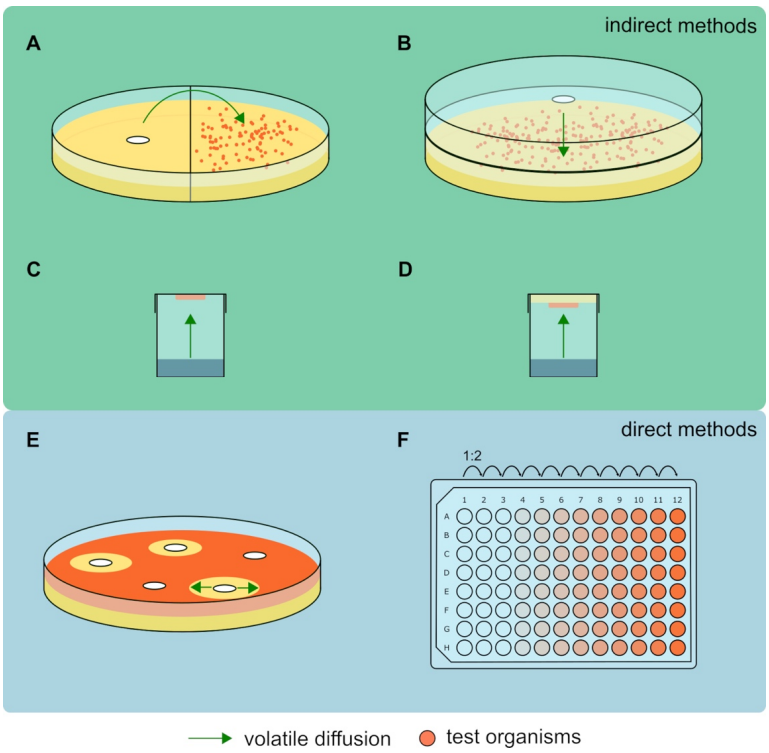

Figure 2. Overview of indirect (**A**–**D**) and direct (**E**,**F**) approaches to test the antimicrobial activity of volatiles. Indirect approaches such as the two-chamber Petri dish (**A**), double plate (**B**), vial (**C**) and AntiBio Vol approach (**D**) compel the volatiles to diffuse through the gas phase to reach the test organisms. In contrast, direct approaches such as the agar diffusion approach (**E**) and the minimal inhibitory test (**F**) allow direct contact between the volatiles and test organisms. Details are described in the main text.

5. Concluding Remarks and Future Perspectives

Recent studies clearly demonstrate the ability of various bacteria to produce antimicrobial volatiles that inhibited the growth of either human or plant pathogens, indicating their antibiotic potential and possible application in agriculture and medicine. The demand for new approaches and compounds is high in both agriculture (due to an EU ban of many chemical pesticides) and healthcare (due to antibiotic resistance and side-effects). As the research of antimicrobial volatiles is a newly developing field, there are still many novel volatile compounds to be discovered and chemically characterized. Individual compounds might be commonly found in many often unrelated strains, while others are restricted only

to a certain group of strains. Usually, mixtures of compounds are released with widely varying concentrations. Yet, their effects on other organisms and their biosynthesis need to be investigated in more detail in the future. Furthermore, the effects of antimicrobial volatiles need to be evaluated on non-target beneficial (micro)organisms. Many fundamental questions about the modes of action of antimicrobial volatiles and possible resistances remain unanswered and need to be investigated in order to advance our basic knowledge in this research field.

Alongside the treatment of pathogens with volatiles alone, another approach is to combine volatiles with common antibiotics. For example, when exposed to the volatile blend of a *Streptomyces* species, *Bacillus subtilis* showed increased sensitivity against several antibiotics [39]. Interestingly, other studies indicate opposite effects. *Bacillus subtilis* exposed to triethylamine showed reduced sensitivity to tetracycline [94]. However, a selective combination of volatiles and common antibiotics may be a successful tool against resistant bacteria in future.

Volatiles may be also used for fast and reliable detection of pathogen infections as pathogens emit volatiles as well. For example, by analyzing the volatile profile of plant pathogenic fungi and oomycetes, we revealed that each isolate emits a specific blend of volatiles [24]. Another study investigated the breath volatile profile of swine infected with Influenza A, resulting in volatiles that could be related to the infection [95]. Other studies worked with human cells lines (lung epithelium) and combined those with *Pseudomonas aeruginosa* causing ventilator-associated pneumonia showing likewise volatiles that could potentially be used as biomarkers [96,97]. *Staphylococcus aureus* is a common bacterium infecting children with cystic fibrosis and a recent study detected this pathogen in cystic fibrosis patients using breath volatile profiles [98].

In agriculture as well volatiles can provide information of early pathogen infractions. Volatiles emitted during the infection of apple plants by bacterial pathogens *Erwinia amylovora* or *Pseudomonas syringae* pv. *syringae* were studied by gas chromatography-mass spectrometry and proton transfer reaction-mass spectrometry. Infected plants showed a disease-specific emission of volatiles, including several bioactive compounds, such as hexenal isomers and 2,3-butanediol [99]. Those approaches are non-invasive, fast, reliable and have the potential to avoid the prophylactic and wrongly use of antibiotics.

Finally, the potential use of volatiles as antimicrobials is often criticized because of their physicochemical properties. In fact, numerous volatiles are liquid at room temperature if not solid and could therefore be solved in appropriate solvents. Furthermore, Avalos suggested the inhaling of volatiles as possible therapy in future [18]. We are convinced that innovative and novel approaches are needed, and antimicrobial volatiles could be a future solution.

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ARTICLE II

ANTIMICROBIAL COMPOUNDS IN THE VOLATILOME OF SOCIAL SPIDER COMMUNITIES

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Antimicrobial Compounds in the Volatilome of Social Spider Communities

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Social arthropods such as termites, ants, and bees are among others the most successful animal groups on earth. However, social arthropods face an elevated risk of infections due to the dense colony structure, which facilitates pathogen transmission. An interesting hypothesis is that social arthropods are protected by chemical compounds produced by the arthropods themselves, microbial symbionts, or plants they associate with. *Stegodyphus dumicola* is an African social spider species, inhabiting communal silk nests. Because of the complex three-dimensional structure of the spider nest antimicrobial volatile organic compounds (VOCs) are a promising protection against pathogens, because of their ability to diffuse through air-filled pores. We analyzed the volatilomes of *S. dumicola*, their nests, and capture webs in three locations in Namibia and assessed their antimicrobial potential. Volatilomes were collected using polydimethylsiloxane (PDMS) tubes and analyzed using GC/Q-TOF. We showed the presence of 199 VOCs and tentatively identified 53 VOCs. More than 40% of the tentatively identified VOCs are known for their antimicrobial activity. Here, six VOCs were confirmed by analyzing pure compounds namely acetophenone, 1,3-benzothiazole, 1-decanal, 2-decanone, 1-tetradecene, and docosane and for five of these compounds the antimicrobial activity were proven. The nest and web volatilomes had many VOCs in common, whereas the spider volatilomes were more differentiated. Clear differences were identified between the volatilomes from the different sampling sites which is likely justified by differences in the microbiomes of the spiders and nests, the plants, and the different climatic conditions. The results indicate the potential relevance of the volatilomes for the ecological success of *S. dumicola*.

Keywords: volatile organic compound, chemical ecology, antimicrobial, *Stegodyphus dumicola*, social arthropods

INTRODUCTION

Organisms use chemicals to exchange information, coordinate their behavior, or protect themselves against pathogens (Chen et al., 1998; Rowan, 2011; Schulz-Bohm et al., 2017). For example, social arthropods depend on chemical compounds, which are vital for communication and other functions mediating a high level of organization. They are able to inhabit extreme environments,

use numerous resources, and finally often outcompete other arthropods (Wilson, 1987; Hölldobler and Wilson, 1990; Fisher et al., 2019). However, a fundamental problem of social arthropods is the elevated risk of acquiring and transmitting pathogens, as their dense colony associations increase the risk of infections and pathogen transmission (Bratburd et al., 2020). The risk of infections has led to a number of adaptations ranging from behaviors that reduce the risk of transmission (Müller and Schmid-Hempel, 1993) to the use of antimicrobial compounds. Antimicrobial compounds can be produced among others by the arthropod hosts themselves (Graystock and Hughes, 2011), symbiotic microorganisms (Musa Bandowe et al., 2009), or surrounding plants (Tariq et al., 2019).

Sociality has also evolved within the class of arachnids (Lubin and Bilde, 2007). *Stegodyphus dumicola* is a social spider species living in large groups in southern and central Africa (Aviles, 1997; Lubin and Bilde, 2007). These spiders build communal nests where reproduction takes place and which protects the spiders against heat/dehydration, UV-radiation, and predators (**Supplementary Figure 1A**; Seibt and Wickler, 1990; Henschel, 1998; Lubin and Bilde, 2007). The nest is surrounded by three-dimensional capture webs used for communal prey capture (Majer et al., 2018). The social lifestyle in spiders comes with elimination of pre-mating dispersal and therefore a strictly inbreeding mating system. Combined with frequent extinction and colonization events, this results in extremely low genetic diversity (Lubin and Bilde, 2007; Settepani et al., 2014, 2016, 2017). Homozygosity in genes within individuals, and low population genetic diversity in for example immune genes is likely to be associated with elevated vulnerability to infections. This substantiates the hypothesis that antimicrobial compounds play an important role in protecting the spider hosts against pathogens.

Due to the complex nest structure, including tightly woven silk structures with multiple narrow tunnels and chambers, volatile organic compounds (VOCs) have a possible importance in pathogen defense. VOCs are carbon-based compounds with molecular masses below 400 Da, high vapor pressures, low boiling points, and lipophilic moiety, properties that imply versatility of these compounds in all terrestrial ecosystems (Rowan, 2011; Schmidt et al., 2015b; Tyc et al., 2015). As opposed to soluble compounds, VOCs can diffuse through air-filled pores in complex ecosystems such as soil and hence do not depend on solvents (Schmidt et al., 2015a). That suggests that VOCs have the potential to reach all the internal surfaces of the spider nest and serve as inhibitors of pathogens from a distance. Antimicrobial VOCs are widely produced by microorganisms, plants, and animals: For example, bacteria isolated from the rhizosphere are known for producing VOCs with antimicrobial properties that can have protective functions for symbiotic plants (Tyc et al., 2015; Ossowicki et al., 2017). Plants use VOCs by themselves in various functions such as preventing microbial infections (Hammerbacher et al., 2019). In termites, volatile pheromones produced by queens, primarily used for communication, were shown to also have antifungal effects, indicating a role in pathogen defense (Matsuura and Matsunaga, 2015). Red fire ants and beetles are also known to use VOCs with antimicrobial properties (Gross et al., 2008; Wang et al., 2015). Here, we propose that VOCs may have a protective antimicrobial function in the nest system of the social spider *S. dumicola*.

The aim of this study is to describe the volatilomes of the spider *S. dumicola* and its nest and web, respectively, and to assess its potential antimicrobial effects. We hypothesized the presence of antimicrobial VOCs in the volatilomes of *S. dumicola*, its nest, and catching web. Next, we investigated whether the spider, the nest, and the capture web emit different VOC blends. Finally, we investigated volatilomes collected at different geographical locations to assess differences between the VOC blends. For this, we analyzed and compared the volatilomes from spider nests, catching webs, and the spiders themselves at three sampling sites in a north-south gradient in Namibia. Furthermore, we tested a selection of identified VOCs as pure compounds for their antimicrobial activity against microbial pathogens of spiders and humans.

MATERIALS AND METHODS

Sampling Sites

The volatilome samples were taken at three different sampling sites in Namibia between 8th–26th February, 2019 (**Supplementary Figure 2**). The locations were close to the Etosha National Park ("Otavi"; S19.47, E17.19), the capital ("Windhoek"; S22.57, E17.21), and a small town ("Stampriet"; S23.74, E18.19). Otavi and Stampriet are at an altitude of ∼1,300 m and Windhoek of ∼2,000 m. At each sampling site five nests were analyzed and defined as biological replicates. The maximal distance between the used spider nests were 150 m in Otavi, 300 m in Windhoek, and 1,200 m in Stampriet (**Supplementary Figure 2** inserts). Otavi is a humid sampling site in the North, Windhoek is mountainous, and Stampriet is because of its closeness to the Kalahari Desert on average the warmest region.

In Otavi the nests were located in plants of the species *Combretum imberbe*, *Acacia mellifera*, *Ziziphus mucronata*, and *Grewia flava*; in Windhoek in *Acacia hereroensis*, *Acacia mellifera*, and *Acacia hebeclada*; in Stampriet in *Acacia nebrownii*. We empirically observed the highest density of plants in Otavi and lowest in Stampriet.

The humidity and temperature data during the volatilome trapping were measured using iButton® logger every 300 s (Type DS1923, Maxim Integrated, San Jose, California, United States). The temperature and humidity data inside the nests during the VOC samplings revealed the nests in Otavi as warmest and less humid and the nests in Windhoek as less warm and most humid (**Supplementary Figure 3**).

Volatilome Trapping

Polydimethylsiloxane (PDMS) tubes (internal diameter 1 mm, external diameter 1.8 mm, Carl Roth, Karlsruhe, Germany) were cut into 5 mm pieces and threaded on needles. The tubes were fully covered with acetonitrile/methanol (4/1, v/v) and incubated for 3 h at room temperature. Subsequently they were dried under N_2 flow (5 l/min) and heated up to 210°C for 1.5 h under He flow

(5 l/min). Glass vials for storing the tubes until usage and needles for fixation at the spider nests and webs were cleaned similarly.

The PDMS tubes were used to trap volatile organic compounds (VOCs) from the spider nests, catching webs, and isolated living spiders. For trapping VOCs from the nests, five tubes were fixed with needles at the nest surfaces (**Supplementary Figure 1B**). For trapping VOCs from the catching webs, five PDMS tubes were threaded on needles to increase the surface and stuck to the catching webs in an approximately 30 cm diameter around the nests. PDMS tubes in sterile, open Petri dishes in 1 m distance to the nests were used as controls. The nests and webs were irritated as little as possible before, during, and after VOC trapping to stress the spiders as little as possible. No spiders or material were removed before trapping. VOCs from spiders were trapped by keeping five spiders from each nest together with five PDMS tubes in a sterile Petri dish. The controls for spider VOCs were closed Petri dishes with five PDMS tubes. All PDMS tubes were left for 30 min and immediately stored in closed glass vials at −20°C until VOC analysis. One nest (together with its catching web and spiders) was defined as one replicate. Per sampling site 3–5 replicates were taken.

GC/Q-TOF Analysis

The VOCs were released from the PDMS tubes using an automated thermodesorption unit (Unity TD-100, Markes International, Llantrisant, United Kingdom) at 280°C for 8 min with a He flow of 50 ml/min. The VOCs got cold trapped at −10°C on a Tenax trap (Markes International, Llantrisant, United Kingdom) and released at 300°C within 10 min. A split ratio of 1/4 was used. The VOCs were transferred (195°C transfer line) to the Agilent 7890B GC (Agilent Technologies, Inc., Santa Clara, CA, United States) with an DB-5 ms ultra inert column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness, 122–5,532, Agilent Technologies, Inc., Santa Clara, CA, United States) and a run time of 35.6 min. The temperature program was set to 39°C for 1 min followed by heating up to 315°C with 10°C/min and holding for 7 min. The MS (280°C transfer line) was performed with Agilent 7200AB Q-TOF at 70 eV in electron ionization mode with a source temperature of 230°C. Mass spectra were recorded in full-scan-mode (m/z 30–400, 4 scans/s, 2 GHz Extended Dynamic Range).

For calibration of the retention index 1 μl alkane standard solution of C_8–C_{20} (40 mg/l in Hexane; 04070-5ML; Merck, Darmstadt, Germany) was spiked on an empty Tenax trap and measured as described above. The presence of acetophenone, 2-decanone, 1-decanal, 1,3-benzothiazole, 1-tetradecene, and docosane (1 μg/ml in MeOH; all purchased by Merck, Darmstadt, Germany) in the volatilomes was confirmed by measuring pure standard compounds on the same way.

GC/Q-TOF Data Processing

For GC/Q-TOF data processing the raw data were exported as content definition file (CDF) and imported into MZmine (Version 2.20;© Copyright 2015; Pluskal et al., 2010). Mass detection, chromatogram building, deconvolution (local minimum algorithm), and peak alignment (RANSAC, random sample consensus) were performed using MZmine (for detailed parameters are provided in **Supplementary Table 1**). The peak lists were exported as comma-separated values (CSV) files. The files were uploaded to MetaboAnalyst (Version 4.0, Xia Lab, Montreal, Canada; Xia and Wishart, 2011), filtered (interquartile range), transformed (log transformation), and scaled (auto) before statistical tests were performed. Statistically significant differences between the samples and controls were identified by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD). Furthermore, a mass feature must be found in at least 4 of 5 or 3 of 4 of the biological replicates to get valued as such.

Compound identification was performed using AMDIS 2.72 (National Institute of Standards and Technology, United States) based on retention index comparison and mass spectrum comparison to three libraries providing more than 1.4 million spectra namely NIST 2014 V2.20 (National Institute of Standards and Technology, Gaithersburg, Maryland, United States), Wiley 7th edition spectral libraries (Wiley, Hoboken, New Jersey, United States), and an internal library of NIOO-KNAW (Netherlands Institute of Ecology, Wageningen, The Netherlands). The retention index tolerance was ± 10 and the minimum mass spectrum match was 600 ‰. If a mass feature complied both criteria, a visual comparison of the mass spectra was performed, and designated as "tentatively identified." Additionally, the retention indices and mass spectra of the "identified" compounds were compared to pure standard compounds measured in the same GC/Q-TOF system. Unknown mass features were assumed as different ones when the retention indices differed by > 6.

Antimicrobial Test of Pure VOCs

Five VOCs identified using pure standard compounds, namely acetophenone, 2-decanone, 1-decanal, 1,3-benzothiazole, and 1-tetradecene, were tested as pure compounds for their antimicrobial activity. The compounds were selected based on literature indicating their high antimicrobial activity (**Supplementary Table 2**). We used *Bacillus thuringiensis* (DSM 2046), *Staphylococcus aureus* (DSM 799), *Escherichia coli* (DSM 787), and *Candida albicans* (DSM 10697) as test strains to cover all, a suggested spider pathogen, Gram positive and negative bacteria, as well as a yeast. The latter three strains are common human pathogens and were chosen as model organisms. All strains were bought at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Before use the strains were pre-cultured overnight at 37°C on Mueller Hinton Agar II (2.0 g/l beef heart infusion, 17.5 g/l acid casein hydrolysate, 1.5 g/l starch, 17.0 g/l agar; Becton Dickinson, Franklin Lakes, New Jersey, United States).

The test strains were diluted to an OD_{600} of 0.1 in Mueller Hinton Broth II (17.5 g/l casein acid hydrolysate, 3 g/l beef extract, 1.5 g/l starch; Sigma-Aldrich, St. Louis, Missouri, United States). 10 ml of the prepared cell solution was transferred into a 100 ml-Erlenmeyer flask and test compounds, were added at a final concentration of 30 mM. The flasks were

sealed immediately with aluminum foil and wrapping film and incubated (37°C, 150 rpm). After 24 h the OD_{600} was measured and compared to the negative control without added compounds (*t*-test, $p \leq 0.05$). Three biological replicates for each test strain in combination with each test compound were performed.

Additionally, an agar diffusion test was performed based on the protocol of Hudzicki (2009). The test strains were diluted to an OD_{600} of 0.125 in NaCl (0.9%) and spread on Mueller Hinton II agar plates (Becton Dickinson, Fraklin Lakes, New Jersey, United States) using cotton swabs. Five microliters of the pure compounds were pipetted on empty cotton disks (6 mm diameter) and placed on the plates before sealing with Parafilm®. Empty cotton disks were used as negative controls. For positive controls cotton disks with gentamicin (Sensi-Disk™, 10 U, Becton Dickinson, Franklin Lakes, New Jersey, United States) were used for bacterial strains and amphotericin B (ROTI® Antibiotic Disks, 100 U, Carl Roth, Karlsruhe, Germany) for the yeast. After incubation (24 h, 37°C) the zones of inhibition (ZOI) were measured.

Identification of Differences Between Volatilomes

To compare the volatilomes between the nests, catching webs, and spiders, and between the sampling sites, partial least squares discriminant analysis (PLS-DA) plots and Euler plots were made. PLS-DA plots were based on all mass features (including relative intensities) and made using MetaboAnalyst (Version 4.0, Xia Lab, Montreal, Canada; Xia and Wishart, 2011). Euler plots were made based on all compounds (**Supplementary Table 2**) VOCs using RStudio (RStudio, Inc., Version 1.2.5033).

RESULTS

Antimicrobial VOCs in the *Stegodyphus dumicola* Volatilome

The analyses of the volatilomes of all sampling sites resulted in the tentative identification of 53 compounds ranging from C_4 up to C_{24} (**Table 1** and **Supplementary Table 2**). Most of the identified VOCs were pure hydrocarbons (41%) or contained oxygen (51%). Eight percent of compounds contained nitrogen, sulfur, or halogens. The VOCs belonged to various chemical classes. Most common were alkanes, carboxylic acids, alcohols, benzenes, ketones, alkenes, aldehydes, and terpenoids. One hundred forty-six compounds could not be tentatively identified by mass spectra comparison with databases and are therefore listed as unknown (**Supplementary Table 2**). Antimicrobial activity was assigned to 21 of the 53 tentatively identified VOCs (**Table 1**) based on published data for the pure compounds or mixtures such as essential oils containing the compound (references in **Supplementary Table 2**). Most of those compounds are known for both antibacterial and antifungal activities. By analyzing pure standard compounds, we confirmed the presence of acetophenone, 2-decanone, 1-decanal, 1,3-benzothiazole, 1-tetradecene, and docosane (**Supplementary Figure 4**).

Effect of Antimicrobial VOCs on Pathogens

A selection of pure compounds found exclusively in the nest volatilome (acetophenone and 2-decanone), the nest and web volatilome (1-decanal and 1,3-benzothiazole) and in the spider volatilome (1-tetratdecene) were tested for their antimicrobial activities against a spectrum of microbial pathogens. All five tested compounds displayed antimicrobial activity (**Figure 1**). Acetophenone, 2-decanone, and 1,3-benzothiaziole significantly inhibited all four tested pathogens. 1-Tetradecene and 1-decanal significantly inhibited only three pathogens, namely *B. thuringiensis*, *S. aureus*, and *C. albicans* but not *E. coli*. 1-Decanal even increased the growth of *E. coli* albeit not significantly. The spider pathogen *B. thuringiensis* was significantly inhibited by all five compounds. Additionally, we tested the antimicrobial activity of the compounds using an agar diffusion test resulting in similar inhibitions (**Supplementary Figure 5**).

Comparison of Nest, Web, and Spider Volatilomes

At all sampling sites, more VOCs were detected in the nest and web samples as compared to the spider samples (**Figure 2** and **Supplementary Figure 6**). The nest and web volatilomes from the site Otavi shared many common VOCs (63 in total, **Figure 2A**). Only eight unknown VOCs were commonly found in the nest, web, and spider volatilomes. In Windhoek we found nearly twice the number of VOCs in the nest compared to the web and spider volatilomes (**Figure 2B**). Two unknown VOCs were common and detected in the nest, web, and spider volatilomes. In Stampriet, similar to Otavi, the nest and web shared a high number of VOCs (**Figure 2C**). Only one compound was found in the combined volatilome of nest, web, and spider and identified as oxymethylencampher.

Comparison of Volatilomes From Different Climatic Regions

Analyzing the volatilomes of the three sampling sites using PLS-DA indicated that the nest volatilomes from the three sampling sites clearly differed from each other, with the largest differences being observed between Otavi and Windhoek (**Figure 3**). The confidence regions of Otavi and Stampriet showed only small overlays (**Figure 3A**). The web volatilomes separated in a similar way with a small overlayed area between Stampriet and Windhoek (**Figure 3B**). The confidence regions of the spider VOCs of Stampriet overlayed in large parts with those of Windhoek, whereas the Otavi volatilome was clearly separated from the other regions (**Figure 3C**).

The Euler-plots based on all identified and unknown VOCs indicated that the Otavi volatilomes show the highest number of VOCs in the nest, and web volatilomes, followed by Stampriet, and Windhoek (**Figure 4** and **Supplementary Figure 6**). The spider volatilomes contained the highest number of VOCs in Otavi and the lowest in Stampriet. The nest volatilomes of the three sampling sites shared 12 common VOCs, and the web 11 common VOCs. The spider volatilomes did not contain common

TABLE 1 | List of identified compounds in the nest (N), web (W), and spider (S) volatilomes of the different sampling sites Otavi, Windhoek, and Stampriet with known antimicrobial activity.

					Otavi			Windhoek			Stampriet		
Compound	**Class**	**Molecular Formular**	**Antimicrobial Activity**	**Compound Origin**	**N**	**W**	**S**	**N**	**W**	**S**	**N**	**W**	**S**
1-Heptanal	Aldehyde	$C_7H_{14}O$	b, f	p						×			
2-Ethylhexanol	Alcohol	$C_8H_{18}O$	b, f	p, f				×	×		×	×	
Acetophenone	Ketone	C_8H_8O	b	?	×			×					
1-Non-anal	Aldehyde	$C_9H_{18}O$	b, f	p	×						×		
Levomenthol	Alcohol	$C_{10}H_{20}O$	b, f	p		×							
2-Decanone	Ketone	$C_{10}H_{20}O$	b, f	p, b				×			×		
Dodecane	Alkane	$C_{12}H_{26}$	b, f	p, b							×		
1-Decanal	Aldehyde	$C_{10}H_{20}O$	b, f	p	×	×							
1,3-Benzothiazole	Benzothiazole	C_7H_5NS	b, f	p	×	×		×					
1-Undecanol	Alcohol	$C_{11}H_{24}O$	b	?								×	
1-Dodecene	Alkene	$C_{12}H_{24}$	b	p							×		
1-Tridecene	Alkene	$C_{13}H_{26}$	b	p, a	×	×		×	×		×		
2-Ethyl-3-hydroxyhexyl 2-methylpropanoate	Carboxylic Acid	$C_{12}H_{24}O_3$	b, f	p	×								
1-Tetradecene	Alkene	$C_{14}H_{28}$	b, f	p, f			×						
1-Dodecanal	Aldehyde	$C_{12}H_{24}O$	b, f	p							×		
Nerylacetone	Ketone	$C_{13}H_{22}O$	b	p	×	×						×	
1-Dodecanol	Alcohol	$C_{12}H_{26}O$	b	p		×	×	×			×		
Pentadecane	Alkane	$C_{15}H_{32}$	b, f	a, i							×	×	
Myristic acid	Carboxylic Acid	$C_{14}H_{28}O_2$	b, f	p	×						×		
Heneicosane	Alkane	$C_{21}H_{44}$	b	p							×	×	
Docosane	Alkane	$C_{22}H_{46}$	b, f	p							×	×	

The antimicrobial activity of the VOCs was shown against bacteria (b) and/or fungi (f). The VOCs originated from plants (p),insects (i), algae (a), bacteria (b), and/or fungi (f). Antimicrobial activities and origins are based on literature data (references in ***Supplementary Table 2****). Detection of VOCs in the samples is indicated by " × ".*

VOCs among sites. 1-Methoxy-2-propanol and 1-tridecene were detected in all nests. 2,6,11-Trimethyldodecane was detected in all web volatilomes. The other shared VOCs remain unknown.

DISCUSSION

The results of our study revealed the presence of numerous VOCs with antimicrobial function in the *S. dumicola* volatilomes. Overall, around 40% of the identified VOCs in the volatilomes of *S. dumicola* exhibit antimicrobial activity based on literature (references in **Supplementary Table 2**). In the present study, several pure VOCs, namely acetophenone, 1-tetradecene, 1-decanal, 2-decanone, and 1,3-benzothiazole, displayed antimicrobial activity against a range of spider and human pathogens. The suggested spider pathogen *B. thuringiensis* (Keiser et al., 2016) and the human pathogens *S. aureus, E. coli*, and *C. albicans* (Peterson, 2009; Canet et al., 2018) were significantly inhibited by most of the tested VOCs, indicating a possible protection of *S. dumicola* against bacteria (Gram-positive and -negative) and yeasts. Our results are in line with several other studies that tested the antimicrobial activity of these compounds as pure compounds or extracts with concentrations between 0.55–43.8% (Palic et al., 2002; Shi et al., 2010; Tayung et al., 2011; Li et al., 2012; Liu et al., 2012; Guleria et al., 2013; Kazemi and Sharifi, 2017). For example, acetophenone and 2-decanone as pure compounds showed both antibacterial and -fungal activity (Rajabi et al., 2005; Sivakumar et al., 2008; Zheng et al., 2013; Jayakumar et al., 2020). The appearance of other antimicrobial VOCs was shown in other arthropod systems, for example in termites (Chen et al., 1998; Matsuura and Matsunaga, 2015), beetles (Gross et al., 2008), and ants (Wang et al., 2015). We found between 7 and 14 antimicrobial VOCs at each sampling site indicating likewise a high potential of the *S. dumicola* volatilomes in pathogen protection.

The origin of the VOCs in *S. dumicola*'s volatilome appears to be diverse—in principle, the spiders themselves, symbiotic microorganisms, prey (and their microbiota), the plants in which the nests are located, and even passing or hostile animals could influence the volatilomes (**Figure 5**). Most of the VOCs with known antimicrobial activity identified in this study were previously reported from essential oils of plants, but some also from fungi, bacteria, algae, or even insects (**Table 1**). The lowest number of VOCs was found in the volatilomes of isolated spiders, whereas relatively more VOCs were detected in the nest and web volatilomes. Furthermore, the spider volatilomes only shared a small number of VOCs with the nest and web volatilomes, whereas the nest and web shared many common VOCs. Particularly the nest may provide a source for a diverse community of microbes as plant material and exoskeletons are

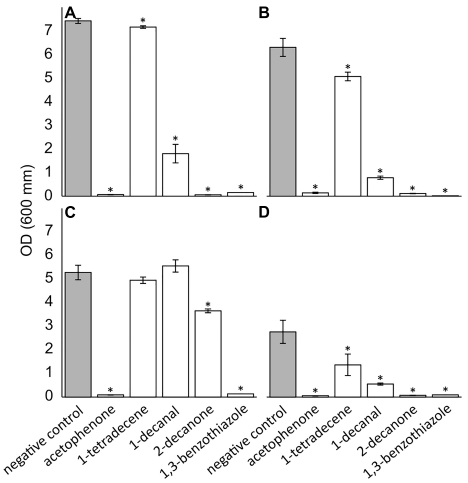

FIGURE 1 | Antimicrobial effect of pure VOCs identified in the volatilomes of *Stegodyphus dumicola*. The compounds were tested at 30 mM against *Bacillus thuringiensis* **(A)**, *Staphylococcus aureus* **(B)**, *Escherichia coli* **(C)**, and *Candida albicans* **(D)**. The error bars show the standard deviation. The * indicate significant decreases in OD_{600} after 24 h incubation; *t*-test, $p \leq 0.05$; $n = 3$.

incorporated in the silk structure providing potential substrates for microbes. As the capture web is much more exposed to wind compared with the nest, we expected less VOCs in the web volatilomes than in the nest volatilomes, however, this was only the case in Windhoek.

A likely origin of VOCs is the *S. dumicola* nest microbiome. Although diverse and differing in composition and relative abundance from nest to nest, four bacterial genera (*Curtobacterium, Modestobacter, Sphingomonas, and Massilia*) and four fungal genera (*Aureobasidium, Didymella, Alternaria, and Ascochyta*) were found in all investigated *S. dumicola* nests and thus form a core nest microbiome (Nazipi et al., 2021). Currently, we cannot link specific VOCs to specific genera. However, the microbiome is the source of both soluble (Currie et al., 1999; Chouvenc et al., 2013; Mendes et al., 2013) and volatile (Musa Bandowe et al., 2009) antimicrobial compounds in other social arthropods, and some of the VOCs we identified in the spider nests are produced by microorganisms. For example, 2-decanone was found in the volatilomes of three *Bacillus* species and showed antifungal activity (Yuan et al., 2012; Zheng et al., 2013; Che et al., 2017; Jayakumar et al., 2020). Furthermore, 1-tetradecene was antimicrobial against numerous bacteria and fungi (Tayung et al., 2011). Another probable origin of the VOCs might be the arthropods themselves. A study identified pentadecane, an antimicrobial compound (Ozdemir et al., 2004; Hussain et al., 2017), in extracts of the head and gaster from argentine ants (Cavill and Houghton, 1974), and we identified the same compound in spider nests and webs in Stampriet. Pentadecane might have originated from dead prey or even hostile ants, even when we didn't observe the latter during field work. The spiders themselves may be a source of the volatilome. For example, it was shown that the cuticular profile of *Stegodyphus lineatus* contains several linear and branched alkanes (Grinsted et al., 2011) and myristic acid plays a role in sexual signaling in *Tegenaria* spp. (Trabalon et al., 1997). Most of the antimicrobial VOCs found in the volatilomes were identified in essential oils of plants, which contain volatile and non-volatile compounds, suggesting that the plants in which the spiders build their nests may influence the nest volatilome. For example, triterpenes extracted from *Combretum imberbe* and *Acacia mellifera* showed antibacterial activity (Angeh et al., 2007; Mutai et al., 2009a). The majority of the plant species in which the spider nests were located in, are known for their antibacterial, antifungal, and/or antiviral activity, even when detailed studies on the chemical compositions are lacking (Masoko et al., 2007, 2010; Peloewetse et al., 2008; Mutai et al., 2009b; Arbab et al., 2015; Lamola et al., 2017; Shikwambana and Mahlo, 2020). All in all, we found several VOCs in the nest, web, and spider volatilomes. The highest diversity was found in the nest and web samples even though the spiders are likely exposed to a mixture of all VOCs present in the spider nest ecosystem. It is likely that the volatilomes are produced communally by bacteria, fungi, plants, and the spiders, respectively, even though we have only little hints to concrete origins yet (**Figure 5**).

The PLS-DA analyses show clear differences between the three sampling sites for each of the analyzed volatilomes (nest, web, spider). The Euler plots support that finding, as the majority of the VOCs (approx. 94%) were only found at one or two locations. There are various potential factors influencing the volatilomes. The nest microbiomes of *S. dumicola* were shown to differ significantly between different sampling sites, even though there is a core microbiome on genus level (Nazipi et al., 2021). A study investigating the spider microbiome of *S. dumicola* (endo- and exosymbionts) showed significant differences even between spiders from different nests from the same sampling site (Busck et al., 2020). Therefore, differences of the nest and spider microbiomes between the different geographic sites might contribute to the differences between the volatilomes found in the present study. The microbiome itself can be shaped by external factors like temperature, humidity, prey, soil, and plant species (Reese and Dunn, 2018). We found clear differences in temperature, humidity, and plant species between the sampling sites, which also goes in line with the differences between the volatilomes. The quantity and quality of the prey is also likely influenced by the location and climate (Majer et al., 2013, 2018). Furthermore, the plants themselves are a potential VOC source (Hammerbacher et al., 2019) and differed clearly between the sampling sites with a much higher plant density in Otavi compared to Windhoek and Stampriet (own observation). It was also shown that the production of essential oils by plants is decreased with increasing altitude (Haider et al., 2009; El-Jalel et al., 2018) which is in line with our results. Thus, the lowest number of VOCs was found at the highest altitude (Windhoek). In contrast to the spider microbiome, it was shown

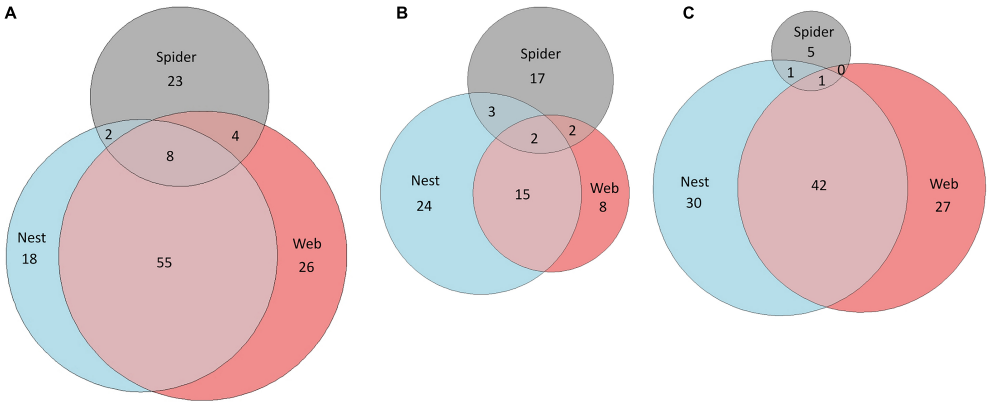

FIGURE 2 | Comparison of the nest, web, and spider volatilomes using Euler diagrams. The diagrams are based on all identified and unknown VOCs from Otavi **(A)**, Windhoek **(B)**, and Stampriet **(C)**. The areas are proportional to the number of VOCs.

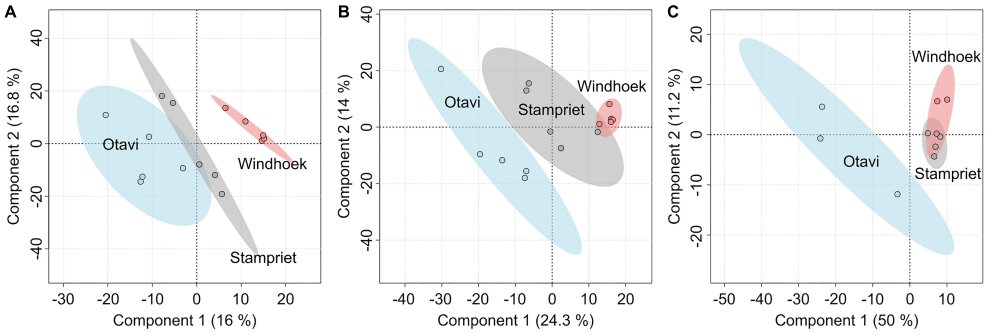

FIGURE 3 | Comparison of the volatilomes of the sampling sites Otavi, Windhoek, and Stampriet using partial least square discriminant analyses (PLS-DA). The diagrams are based on all identified and unknown VOCs of the nests **(A)**, webs **(B)**, and spiders **(C)**. The semi-transparent colored areas show the 95% confidence regions.

that *S. dumicola* shows little genetic differences between sampling sites (Settepani et al., 2017), which makes the spiders themselves very unlikely as an influencing factor for the differences between locations. Even though most of the VOCs were not shared between the sampling sites, some VOCs were present at all sites which suggests a site-independent "core volatilome" of *S. dumicola*. 12 VOCs were found in the nest volatilomes of all sampling sites and 11 VOCs in all web volatilomes. Two tentatively identified VOCs, namely 1-methoxy-2-propanol and 1-tridecene, were found in all nest volatilomes and 2,6,11-trimethyldodecane and 1-tridecene in all web volatilomes. None of these compounds is known from other social insect or spider systems but 1-tridecene showed antibacterial activity (Kumar et al., 2011; Satmi and Hossain, 2016) and 1-dodecanol, a compound we found at all sampling sites in the nests or webs, is well known for its insecticidal (Tabanca et al., 2014) and antibacterial activity (Togashi et al., 2007; Vairappan et al., 2012). It is conceivable that this compound may protect *S. dumicola* against hostile insects like ants or wasps, but also against bacterial pathogens (**Figure 5**).

In conclusion, we found distinct differences between the sampling sites that can be explained with differences in the microbiomes, plant quality and quantity, prey quality, and physical parameters like temperature, humidity, and altitude. We lack information about the *in situ* concentrations of the VOCs, because it is technically impossible to combine minimal invasive, untargeted, and *in situ* volatilome analysis with quantitative conclusions. In soil systems it is known, that

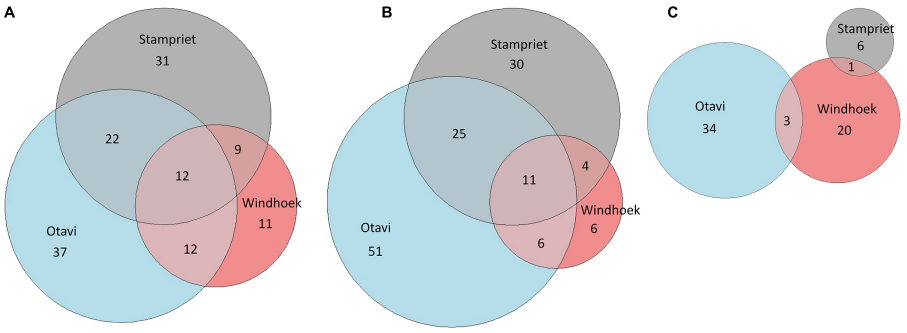

FIGURE 4 | Comparison of the volatilomes of the sampling sites Otavi, Windhoek, and Stampriet using Euler diagrams. The diagrams are based on all identified and unknown VOCs of the nests **(A)**, webs **(B)**, and spiders **(C)**. The areas are proportional to the number of VOCs.

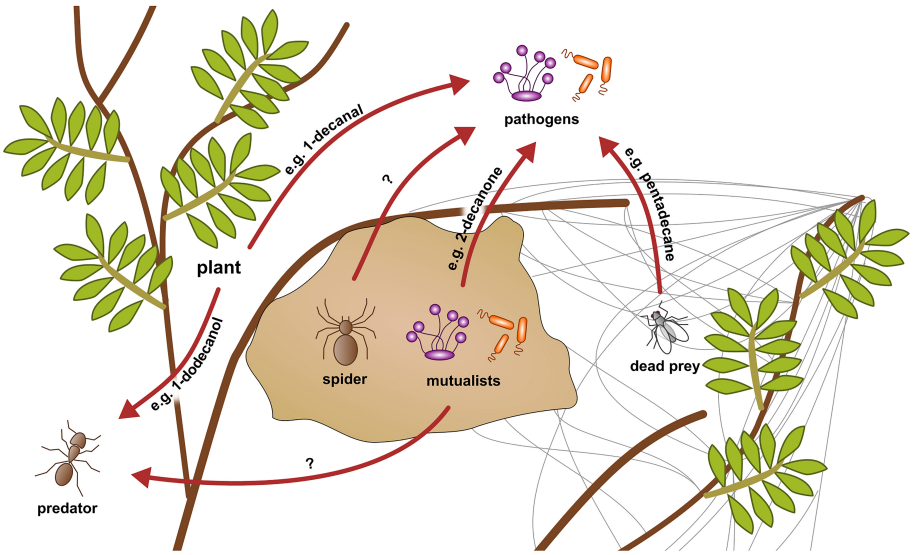

FIGURE 5 | Conceptual scheme of hypothetical VOC interactions in a social spider nest system. VOCs can be originated from the spiders themselves, their microbiome, dead prey, or the plants and inhibit pathogens and predators (red arrows). The example VOCs were found in the analyzed volatilomes of the present study. Their origin and influence are assumed based on literature.

aggregates creates micro-"incubators" influencing microbial life (Rillig et al., 2017). Similar phenomena may be possible in the spider nest ecosystem influencing the growth of certain microorganisms and the accumulation of antimicrobial VOCs. Nonetheless, we found hints for a core volatilome that may be important for the ecological success of *S. dumicola*. Overall, our study revealed that the volatilomes of *S. dumicola* contain numerous VOCs with antimicrobial potential that might play a key role in their pathogen defense. Apart from the tentatively identified VOCs the majority of ∼75% remains unknown. Therefore the *S. dumicola* system might contain more antimicrobial VOCs with the potential to protect the spiders and reveal novel classes of antimicrobial compounds. Next to antimicrobial protection VOCs can also play an important role in communication which should be addressed in further studies (Weisskopf et al., 2021).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AL, AS, HZ, ML, PG, and TS designed the study. AL performed the volatilome trapping, GC/Q-TOF data analysis, and the antimicrobial testing, and wrote the manuscript with support from AS, PG, and TB. TS assisted in planning and realization of the fieldwork. HZ performed the GC/Q-TOF analysis. All authors reviewed the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.700693/full#supplementary-material

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1 Data Availability Statement

The dataset for this study can be found in the zenodo.org database under the number 5034289.
DOI: 10.5281/zenodo.5034289

2 Supplementary Material

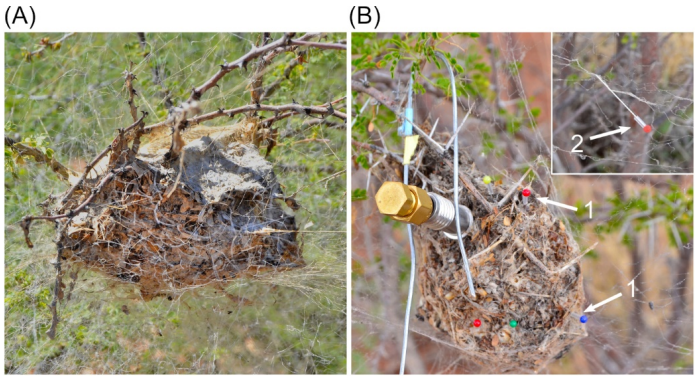

Figure S 1 | Photographs of the nest of *Stegodyphus dumicola* and experimental design of VOC trapping. (A) A nest with a diameter of approximately 20 cm. (B) Polydimethylsiloxane (PDMS) tubes at the nest (1) and the web (2) were used to trap VOCs. The PDMS tubes were combined with needles for better fixation.

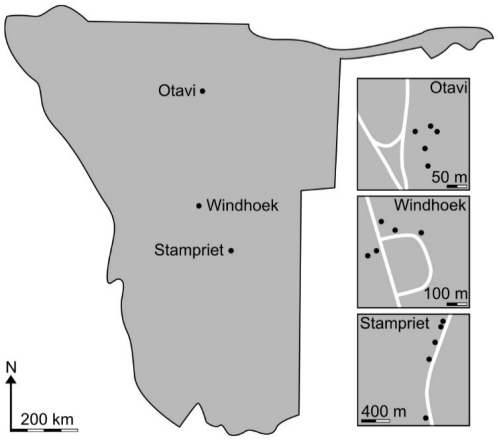

Figure S 2 | Map of Namibia showing the sampling sites. The inserted maps show the distributions of the spider nests at the sampling sites Otavi (S19.47, E17.19), Windhoek (S22.57, E17.21), Stampriet (S23.74, E18.19).

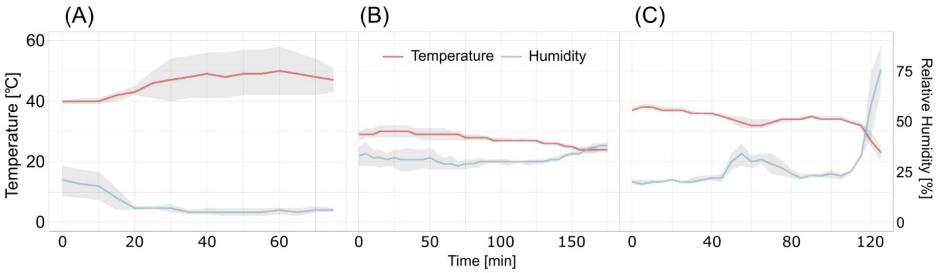

Figure S 3 | Temperature and humidity data of the spider nests during VOC trapping in Otavi (A), Windhoek (B), and Stampriet (C). The grey shadows indicate the maximum span of the parameter. Otavi/Stampriet $n = 3$; Windhoek $n = 4$

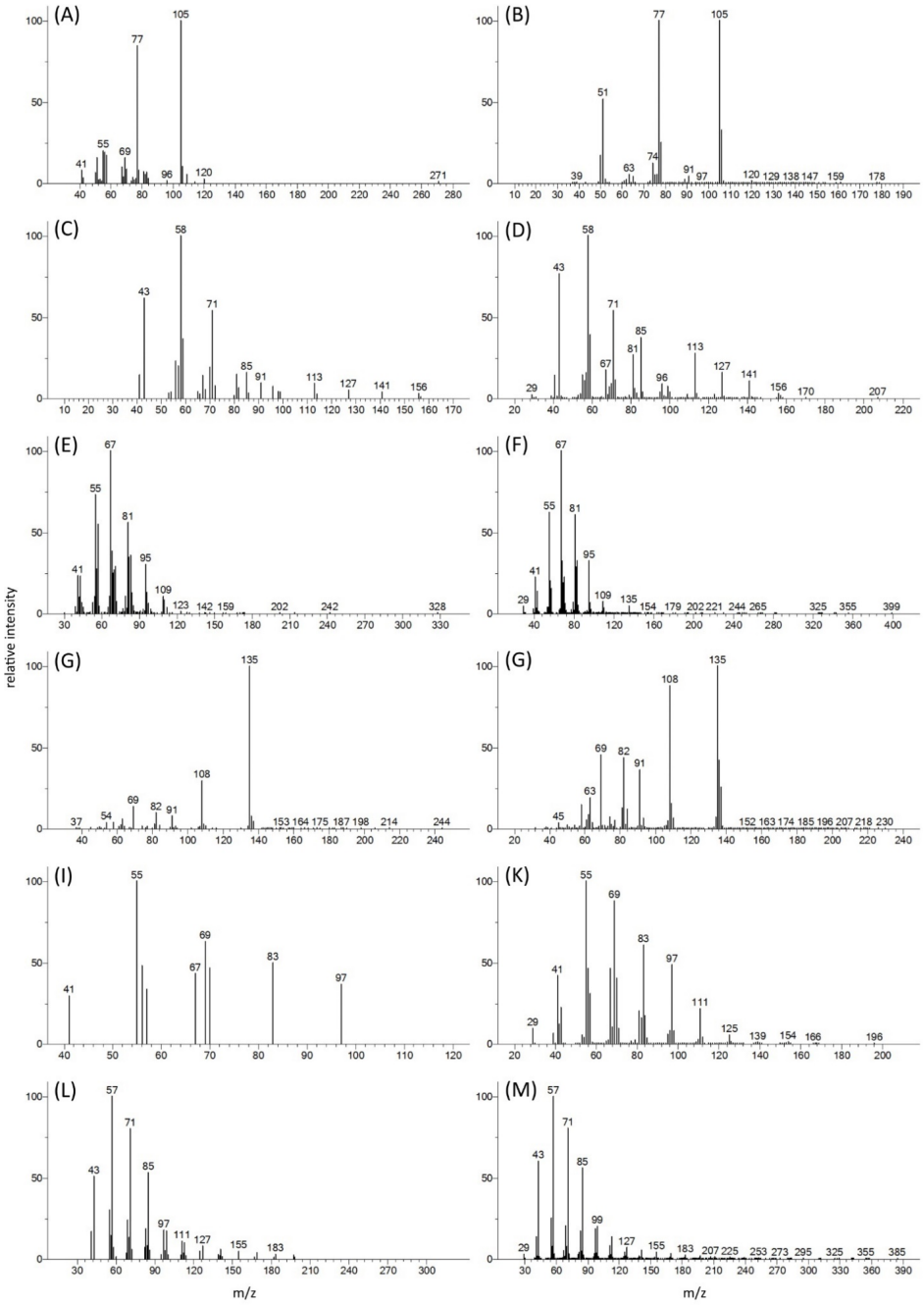

Figure S 4 | Mass spectra of the identified compounds (*in situ* sample/pure analytical standard) acetophenone (A/B), 2-decanone (C/D), 1-decanal (E/F), 1,3-benzothiazole (G/H),

1-tetradecene (I/K), and docosane (L/M).

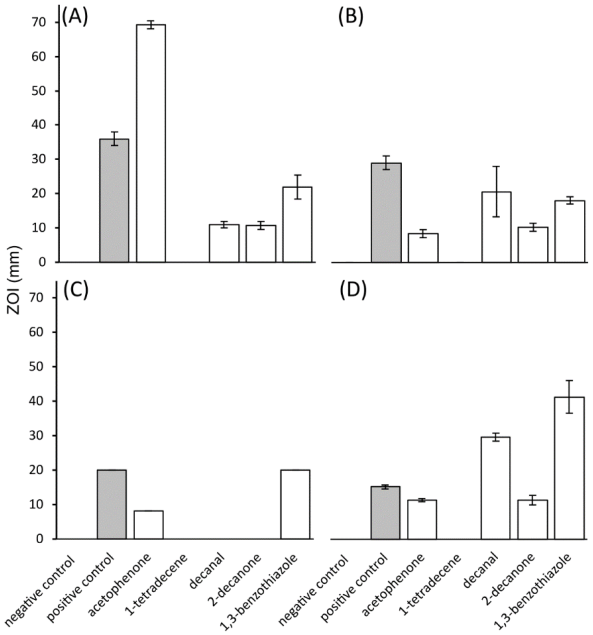

Figure S 5 | Antimicrobial effect of pure VOCs identified in the volatilomes of *Stegodyphus dumicola*. The compounds were tested using the agar diffusion test against *Bacillus thuringiensis* (A), *Staphylococcus aureus* (B), *Escherichia coli* (C), and *Candida albicans* (D). The error bars show the standard deviation. $n = 3$.

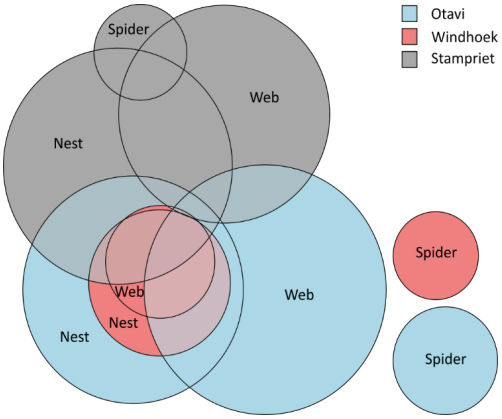

Figure S 6 | Combined comparison between the sampling sites (Otavi, Windhoek, Stampriet) and sampling types (nest, web, spider) using a Euler diagram. The diagram is based on all identified and unknown VOCs. The areas are proportional to the number of VOCs.

Table S 1 | Parameters used for data processing using MZmine. RT – retention time, RANSAC - random sample consensus

Process Step	Parameter [Unit]	Value
Mass Detection	Noise Level	1.00E+02
Chromatogram Building	Minimum Time Span [min]	0.001
	Minimum Height	1.00E+03
	m/z Tolerance [mz / ppm]	1 / 5
Chromatogram Deconvolution (Local Minimum Search)	Chromatographic Threshold [%]	35
	Search Minimum in RT Time [min]	0.03
	Minimum Relative High [%]	10
	Minimum Absolute Height	1.00E+03
	Minimum Ratio of Peak Top/Edge	2
	Peak Duration Range [min]	0-0.5
Peak Aligment (RANSAC)	m/z Tolerance [mz / ppm]	0.001 / 1
	RT Tolerance [min]	0.1
	RT Tolerance After Correction [min]	0.05
	RANSAC Iterations	10,000
	Minimum Number of Points [%]	70
	Threshold Value	1

Table S 2 | Complete list of tentatively identified and unknown compounds found in the volatilomes of *Stegodyphus dumicola* at the sampling sites Otavi, Windhoek, and Stampriet in the nests (N), webs (W), and spiders (S). The presence of a compound in a sample (indicated by "×") was assumed when a significant mass feature compared to the control in 4/5 or 3/4 of the biological replicates were found (ANOVA and Fisher's LSD). The compounds were tentatively identified by comparison of the mass spectra and retention indices with databases. The antimicrobial properties of the compounds were shown in the references. RT – retention time, RI – retention index, match – similarity of the measured mass spectrum with the mass spectrum of the database

						Otavi			Windhoek			Stampriet			
Compound	Class	Molecular Formular	RT [min]	RI	Match [‰]	N	W	S	N	W	S	N	W	S	References
unknown			2.2652	565.6										×	
unknown			2.7692	610.6							×				
Ethyl acetate	Ester	$C_4H_8O_2$	2.7798	611.6	730									×	
unknown			3.0102	632.2		×									
unknown			3.2950	657.6					×						
1-Methoxy-2-propanol	Alcohol	$C_4H_{10}O_2$	3.2952	657.6	857	×			×	×		×			
unknown			3.3908	666.1										×	
unknown			3.6983	693.6								×	×		
Pyridine	Pyridine	C_5H_5N	4.1140	730.7	916				×	×					
unknown			4.1189	731.2		×	×								
unknown			4.6317	776.9		×	×								
unknown			5.4195	832.5		×	×								
unknown			5.6408	846.1		×	×								
unknown			6.2306	882.2		×	×								
unknown			6.3599	890.2								×			
1-Heptanal	Aldehyde	$C_7H_{14}O$	6.5595	902.4	654						×				(Wood and Szewczak, 2007; Shi et al., 2010; Cansu et al., 2013; Li et al., 2013)
Hexanoic acid methyl ester	Ester	$C_7H_{14}O_2$	6.9078	923.1	829			×							
4-Methylnonane	Alkane	$C_{10}H_{22}$	7.6139	965.1	798		×								
unknown			7.6288	966.0		×	×		×				×		
unknown			7.7299	972.0								×	×		
4-Methyl-3-pentenoic acid	Acid	$C_6H_{10}O_2$	7.8892	981.5	642							×			
unknown			7.8926	981.7									×		
unknown			8.0599	991.7				×							

1,3-Dichlorobenzene	Benzene	$C_6H_4Cl_2$	8.3774	1010.9	907	×	×		×	×			
unknown			8.5414	1020.9							×		
2-Ethylhexanol	Alcohol	$C_8H_{18}O$	8.6534	1027.8	831				×	×		×	×
unknown			8.9926	1048.6					×	×			
unknown			9.1305	1057.1				×					
unknown			9.2584	1064.9		×	×		×	×			
1-Octanol	Alcohol	$C_8H_{18}O$	9.3361	1069.7	769		×						
unknown			9.3400	1069.9								×	×
Acetophenone	Ketone	C_8H_8O	9.3447	1070.2	763	×			×				
unknown			9.6690	1090.1							×		
(E)-5-Methyl-4-decene	Alkene	$C_{11}H_{22}$	9.9004	1104.6	651		×						
1-Nonanal	Aldehyde	$C_9H_{18}O$	9.9030	1104.8	739	×						×	
unknown			9.9113	1105.3									×
unknown			10.1685	1122.1		×	×						
unknown			10.2799	1129.4		×	×			×			×
unknown			10.5095	1144.4				×					
unknown			10.6615	1154.3				×					
unknown			10.8792	1168.6				×					
unknown			10.9124	1170.7			×			×		×	
unknown			11.0818	1181.8					×	×		×	×
Levomenthol	Alcohol	$C_{10}H_{20}O$	11.0999	1183.0	794		×						
unknown			11.2267	1191.3			×						×
2-Decanone	Ketone	$C_{10}H_{20}O$	11.2302	1191.5	751				×			×	
unknown			11.2704	1194.1						×			
3,5-Dimethylundecane	Acyl	$C_{13}H_{28}$	11.3576	1199.8	610		×						
4,6-Dimethylundecane	Acyl	$C_{13}H_{28}$	11.3614	1200.1	720								×
Dodecane	Alkane	$C_{12}H_{26}$	11.3618	1200.1	630							×	
unknown			11.3681	1200.6		×		×	×				
1-Decanal	Aldehyde	$C_{10}H_{20}O$	11.4522	1206.4	824	×	×						
unknown			11.5178	1210.9		×			×	×			×

(Kivcak et al., 2007; Pandey and Banerjee, 2014)

(Rajabi et al., 2005; Sivakumar et al., 2008)

(Kivcak et al., 2007; Wood and Szewczak, 2007; Pavithra et al., 2009; Hussain et al., 2017)

(Kaya and Duran, 2018; Gharaibeh et al., 2020; Montenegro et al., 2020; Zhu et al., 2020)

(Guleria et al., 2013; Zheng et al., 2013; Jayakumar et al., 2020)

(Pavithra et al., 2009; Okla et al., 2019; Jayakumar et al., 2020)

(Liu et al., 2012; Kazemi and Sharifi, 2017)

unknown			11.6050	1216.9			×		×			×			
unknown			11.6902	1222.8		×									
unknown			11.7579	1227.4			×								
2,4-Dimethylacetophenone	Phenone	$C_{10}H_{12}O$	11.7644	1227.9	697								×		
unknown			11.8209	1231.8							×	×		×	
1,3-Benzothiazole	Benzothia-zole	C_7H_5NS	11.8901	1236.6	923	×	×		×						(Bondock et al., 2010; Shi et al., 2010; Li et al., 2012)
unknown			11.9854	1243.1		×	×	×	×	×			×		
unknown			12.1096	1251.7		×	×	×	×			×	×		
1,3-Di-tert-butylbenzene	Benzene	$C_{14}H_{22}$	12.1156	1252.1	795						×				
unknown			12.2002	1257.9		×	×	×				×	×		
unknown			12.4475	1275.0		×						×			
2,6,11-Trimethyldodecane	Alkane	$C_{15}H_{32}$	12.4483	1275.1	859		×			×			×		
unknown			12.4535	1275.4					×			×	×		
unknown			12.5696	1283.4		×						×	×		
2-Butyl-1-octanol	Alcohol	$C_{12}H_{26}O$	12.5708	1283.5	727		×								
1-Undecanol	Alcohol	$C_{11}H_{24}O$	12.5800	1284.1	640								×		(Togashi et al., 2007)
1-Dodecene	Alkene	$C_{12}H_{24}$	12.5803	1284.2	660								×		(Roy et al., 2009; Abdelwahab et al., 2010)
unknown			12.7172	1293.6		×	×	×	×	×		×	×		
1-Tridecene	Alkene	$C_{13}H_{26}$	12.8175	1300.5	788	×	×		×	×		×			(Kumar et al., 2011; Satmi and Hossain, 2016)
unknown			12.8186	1300.6				×				×			
unknown			12.9390	1308.9		×	×	×		×	×	×	×		
2,2,4,4,6,8,8-Heptamethylnonane	Alkane	$C_{16}H_{34}$	13.0251	1314.8	635		×								
unknown			13.0507	1316.6		×						×			
unknown			13.2103	1327.6		×	×		×		×	×			
4,6-Dimethyldodecane	Alkane	$C_{14}H_{30}$	13.2312	1329.1	852		×						×		
unknown			13.3473	1337.1		×	×			×		×			
6-Ethyltetralin	Naphtalene	$C_{12}H_{16}$	13.3963	1340.4	684				×						
unknown			13.4385	1343.3				×				×			
5-Methyltridecane	Alkane	$C_{14}H_{30}$	13.4835	1346.5	710								×		
Oxymethylencampher	Ketone	$C_{11}H_{16}O_2$	13.4903	1346.9	637							×	×	×	
unknown			13.5701	1352.4		×	×					×	×		
unknown			13.6573	1358.4				×				×	×		
2-Tert-butyl-4-methylphenol	Phenol	$C_{11}H_{16}O$	13.6620	1358.8	744				×				×		

unknown			13.7703	1366.2			×								
2-Ethyl-3-hydroxyhexyl 2-methylpropanoate	Ester	$C_{12}H_{24}O_3$	13.8539	1372.0	666	×									(Li et al., 2012)
unknown			13.8713	1373.2			×	×			×				
1-Tetradecene	Alkene	$C_{14}H_{28}$	14.0589	1386.1	683			×							(Palic et al., 2002; Tayung et al., 2011)
unknown			14.1905	1395.2							×				
1-Dodecanal	Aldehyde	$C_{12}H_{24}O$	14.2952	1402.4	674						×				(Kubo et al., 2004; Dordevic et al., 2011; Boussalah, 2020)
unknown			14.3209	1404.2				×	×	×					
unknown			14.4879	1415.7			×							×	
unknown			14.7102	1431.0							×	×			
Nerylacetone	Ketone	$C_{13}H_{22}O$	14.7857	1463.3	788	×	×					×			(Zellagui et al., 2012; Kazemi and Sharifi, 2017)
unknown			14.8145	1438.2							×	×			
Dimethylphthalate	Benzene	$C_{10}H_{10}O_4$	14.8155	1438.3	711						×				
unknown			14.9084	1444.7			×	×	×	×	×	×	×		
unknown			15.0489	1454.4		×	×			×		×	×		
1-Dodecanol	Alcohol	$C_{12}H_{26}O$	15.1110	1458.7	809		×	×	×			×			(Togashi et al., 2007; Vairappan et al., 2012)
unknown			15.1796	1463.4		×	×						×		
unknown			15.2999	1471.7		×	×								
unknown			15.3972	1478.4		×	×		×	×			×		
unknown			15.4876	1484.7		×	×	×				×	×		
unknown			15.6492	1495.8				×							
unknown			15.7408	1502.1		×	×	×							
Pentadecane	Alkane	$C_{15}H_{32}$	15.8593	1510.3	791							×	×		(Ozdemir et al., 2004; Hussain et al., 2017)
5-Propyltridecane	Alkane	$C_{16}H_{34}$	15.8602	1510.4	788		×								
unknown			15.8608	1510.4		×						×			
unknown			15.9824	1518.8		×		×				×	×		
unknown			16.0950	1526.6		×	×					×			
1,1,4,5,6-Pentamethylindane	Indane	$C_{14}H_{20}$	16.1394	1529.6	673			×							
unknown			16.1994	1533.7		×	×								
unknown			16.3143	1541.7								×			
unknown			16.4817	1553.2		×	×					×	×		
unknown			16.5991	1561.3				×				×	×		
unknown			16.6885	1567.5		×	×								
unknown			16.8103	1575.9		×	×								

unknown			16.9303	1584.2		×	×		×			×	×	
unknown			17.1503	1599.3		×	×	×					×	
unknown			17.2406	1605.6		×	×		×			×		
unknown			17.3376	1612.2			×			×		×	×	
unknown			17.4680	1621.2		×	×		×			×	×	
unknown			17.6339	1633.1		×	×		×				×	
unknown			17.7798	1642.7		×	×						×	
unknown			17.9592	1655.1			×		×		×			
unknown			18.0542	1661.7					×		×			
1,3-Diisopropylnaphthalene	Naphtalene	$C_{16}H_{20}$	18.0794	1663.4	713	×	×					×	×	
unknown			18.1591	1668.9			×					×		
1,7-Diisopropylnaphthalene	Naphtalene	$C_{16}H_{20}$	18.1612	1669.1	708	×								
unknown			18.2786	1677.1							×			
Propyl laurate	Ester	$C_{15}H_{30}O_2$	18.3904	1684.9	607	×								
unknown			18.3933	1685.1		×	×						×	
unknown			18.4958	1692.1		×	×					×		
unknown			18.6300	1701.4					×			×	×	
unknown			18.8801	1718.6			×							
unknown			19.0014	1727.0		×						×	×	
Isobutyl 2-isobutoxybenzoate	Benzene	$C_{15}H_{22}O_3$	19.0029	1727.1	755		×							
Myristic acid methyl ester	Acid	$C_{15}H_{30}O_2$	19.0829	1732.6	640								×	
unknown			19.1177	1735.0		×	×		×	×				
unknown			19.2503	1744.2					×				×	
Myristic acid	Acid	$C_{14}H_{28}O_2$	19.4584	1758.5	706	×						×		(Agoramoorthy et al., 2007; Altieri et al., 2009; Chen et al., 2019)
unknown			19.4612	1758.7			×					×	×	
unknown			19.5693	1772.4		×								
unknown			19.5694	1766.2		×	×							
unknown			19.8204	1783.5		×	×							
unknown			19.9582	1793.0		×	×		×			×	×	
unknown			20.0821	1801.5								×	×	
unknown			20.1995	1809.6		×	×					×		
unknown			20.3203	1818.0						×			×	
unknown			20.4102	1824.2		×	×		×	×	×	×	×	
unknown			20.6290	1839.2		×	×					×	×	
unknown			20.9175	1859.1		×	×		×					

unknown			21.1084	1872.3		×	×					×	×	
unknown			21.5389	1902.0		×	×					×	×	
unknown			22.4444	1964.4					×					
unknown			22.7400	1984.8		×	×		×	×		×	×	
unknown			22.8425	1991.9			×							
unknown			22.9389	1998.5							×			
unknown			23.1899	2015.9			×				×	×	×	
unknown			23.6183	2045.4		×	×						×	
unknown			23.7388	2053.7		×	×							
unknown			23.8297	2060.0									×	
Heneicosane	Alkane	$C_{21}H_{44}$	24.4592	2103.4	839							×	×	(Boussaada et al., 2008; Kotan et al., 2010)
unknown			24.4600	2103.4			×							
unknown			24.6040	2113.4		×	×							
unknown			25.0016	2140.8							×			
unknown			25.2496	2157.9		×	×		×			×	×	
unknown			25.4398	2171.0		×	×							
unknown			25.7011	2189.0				×						
unknown			25.8890	2202.0		×	×							
Docosane	Alkane	$C_{22}H_{46}$	26.0210	2211.1	728							×	×	(Sinek et al., 2012; Wang et al., 2015)
unknown			26.0337	2212.0					×					
unknown			26.2400	2226.2				×			×			
unknown			26.3792	2235.8				×						
unknown			26.6111	2251.8				×						
unknown			26.7598	2262.1		×	×					×		
Dodecyl 2-pentyl ester sulfurous acid	Sulfurous acid	$C_{17}H_{36}O_3S$	26.7698	2262.7	606								×	
unknown			26.8479	2268.1							×			
unknown			27.1090	2286.1							×	×	×	
unknown			27.2989	2299.2		×	×							
unknown			27.4880	2312.3		×	×							
Methyl copalate	Terpenoid	$C_{21}H_{34}O_2$	27.5995	2320.0	652	×								
unknown			27.6006	2320.0			×	×	×			×	×	
unknown			27.8286	2335.8				×						
unknown			28.0098	2348.3				×						
2-Methyltricosane	Alkane	$C_{24}H_{50}$	28.1795	2360.0	755		×							
unknown			28.1800	2360.0		×								

unknown	28.3610	2372.5					×	×				
unknown	28.4867	2381.1							×	×		
unknown	28.6392	2391.7						×				
unknown	28.9019	2409.8			×							
unknown	29.0184	2417.8						×				
unknown	29.4089	2444.8			×							
unknown	29.5609	2455.2						×				
unknown	29.8282	2473.7	×	×							×	
unknown	29.9189	2479.9			×							
unknown	29.9206	2480.0						×				

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ARTICLE III

ANTIMICROBIAL VOLATILES EMITTED BY MEMBERS OF THE NEST MICROBIOME OF SOCIAL SPIDERS

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Antimicrobial Volatiles emitted by Members of the Nest Microbiome of Social Spiders

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Abstract

Microbial volatiles are ubiquitous, can have antimicrobial properties, and are able to diffuse through complex three-dimensional systems like spider nests making them a promising pathogen protection for social arthropods. Here, we analyzed the volatilomes of five nest microbiome members of the Namibian, social spider *Stegodyphus dumicola*, namely the bacteria *Massilia* sp. IC2-278, *Massilia* sp. IC2-477, *Sphingomonas* sp. IC-11, *Streptomyces* sp. IC-207, and the fungus *Aureobasidium* sp. CE_32, and tested their antimicrobial activity against two putative spider pathogens, namely *Bacillus thuringiensis* and *Purpureocillium lilacinum.* Most nest microbiome members released volatilomes with antibacterial and/or antifungal activities under *in vitro* conditions. The analysis of their volatilomes using GC/Q-TOF revealed that most were unique and contained numerous antimicrobial volatiles. We tested the antimicrobial activity of five pure compounds found in the volatilomes revealing that most of them were both, antibacterial and antifungal. Additionally, we showed the influence of the volatilomes on the antibiotic sensitivity of *B. thuringiensis.* Together, our study shows the antimicrobial activity of the nest microbiome members' volatilomes indicating their potential importance for pathogen protection and that microbial volatiles may offer novel approaches to counter antibiotic resistance.

Introduction

Volatiles of microbial origin may (positively or negatively) influence the growth of other (micro)organisms (Ryu *et al.* 2003; Li *et al.* 2020) and affect their behavior (Robacker and Lauzon 2002; Schulz-Bohm, Martin-Sanchez and Garbeva 2017). Some volatiles have strong antimicrobial activites (Lammers, Lalk and Garbeva 2022) and may explain host-pathogen interactions in arthropod ecosystems. For example, naphthalene in termite nests (*Coptotermes formosanus*) was suggested as protection, against pathogenes and antagonistic arthropods (Chen *et al.* 1998). The antimicrobial activity of ant nest volatilomes (*Solenopsis invicta*) indicates an important role in pathogen defense (Wang *et al.* 2015). Furthermore, the larvae of beetles (*Phratora vitellinae*) secrete antimicrobial volatiles for pathogen protection (Gross *et al.* 2008). Especially the lifestyle of social arthropods, which includes high population densities, and for some species low genetic diversity, causes a remarkable high potential of pathogens spread within a population, posing a risk to all individuals (Schmid-Hempel 1998; Wilson-Rich *et al.* 2009). Antimicrobial compounds produced by microbial symbionts are a feasible defense mechanism to prevent pathogen infections. Whereas soluble antimicrobials are hard to disperse in complex three-dimensional systems like termite or bee nests, volatiles can diffuse through air and stream easily through those systems.

Recently, the *in situ* volatilomes and their antimicrobial potentials of the social spider species *Stegodyphys dumicola* living in southern and central Africa were described (Lammers *et al.* 2021). Fifty-three volatiles in the spider ecosystem could be tentatively identified, of which more than 40 % are known as antimicrobial. While the origins of those volatiles can be diverse, for example, the arthropods themselves, their microbiomes, or essential oils from surrounding plants, a set of 12 volatiles could be detected in all spider nests (Lammers *et al.* 2021). This coincides with an analysis of the nest microbiomes of *S. dumicola*, where four bacterial genera (*Curtobacterium*, *Modestobacter*, *Sphingomonas,* and *Massilia*) and four fungal genera (*Aureobasidium*, *Alternaria*, *Didymella,* and *Aschochyta*) were identified in all nests and thus form a nest core microbiome (Nazipi *et al.* 2021). This leads to the hypothesis that members of the core microbiome produce (part of) the core volatiles, and, more generally, antimicrobial volatiles against spider pathogens.

Here we investigate the antimicrobial potential of the volatilomes of four microbial isolates of the *S. dumicola* nest core microbiome against two putative spider pathogens and analyze the isolates' volatilomes to identify compounds that are potentially responsible for the antimicrobial activity. Additionally, we investigated the volatilome of *Streptomyces* sp. IC-207, which was likewise isolated from *S. dumicola* nests, but does not belong to the core members. However, *Streptomyces* species are well known for their antimicrobial potential including volatiles (Watve *et al.* 2001; Avalos *et al.* 2019).

Materials and Methods

2.1. Microbial Strains, Isolation, and Cultivation Conditions

Five nest microbiome members isolated from Namibian nests of the social spider *Stegodyphus dumicola* were used in this study (**Tab. 1**). The fungus *Aureobasidium* sp. CE_32 and the two *Massilia* strains had been introduced in previous studies (Nazipi *et al.* 2021, submitted). *Sphingomonas* sp. IC-11 and *Streptomyces* sp. IC-207 were isolated by *in situ* cultivation from a spider nest in Otavi, Namibia (-19.47565, 17.19486) in January 2018. Approximately 750 mg of nest material was extracted in phosphate-buffered saline (pH 7.2) and the cell suspension was used to inoculate a modified isolation chip (iChip) as described in detail in Nazipi *et al.* (Nazipi *et al.* submitted). The inoculated iChip was inserted into the nest, left for two weeks, and then transported inside the nest to Aarhus University, Denmark, for disassembly, after which bacterial colonies were streaked to purity on nutrient agar (Scharlau) and classified by Sanger sequencing of their 16S rRNA genes (Nazipi *et al.* submitted).

The putative, fungal spider pathogen *Purpureocillium lilacinum* strain CE_55 (below *P. lilacinum*) was isolated on potato dextrose agar (24 g L^{-1} potato dextrose broth (Sigma-Aldrich), 15 g L^{-1} agar, pH 5.1 ± 0.2) from the surface of a *S. dumicola* spider. The spider was rubbed onto a potato dextrose agar plate supplemented with ampicillin (1 µl mL^{-1}). After five days at 30°C, colonies were streaked to purity on potato dextrose broth and identified by Sanger sequencing (Nazipi *et al.* 2021). The ITS region of isolate CE_55 was 99.59 % identical to *Purpureocillium lilacinum* ATCC 10114 and its partial 28S rRNA gene was 100 % identical. The Genbank accession number of isolate CE_55 is OM189527. The putative, bacterial spider pathogen *Bacillus thuringiensis* ATCC 10792^T was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Table 1. *S. dumicola* nest microbiome members used in this study.

Strain	Best BLAST match, % identity	Genbank accession no.	Reference
Aureobasidium sp. CE_32	*A. melanogenum,* 99.16% (ITS), 100% (28S rRNA)	MT448825.2	Nazipi *et al*. 2021
Massilia sp. IC2-278	*M. brevitalea* byr23-80, 100% (16S rRNA)	MT803700.1	Nazipi *et al*. submitted
Massilia sp. IC2-477	*M. agri* K-3-1, 100% (16S rRNA)	MT803791.1	Nazipi *et al*. submitted
Sphingomonas sp. IC-11	*S. dokdonensis* DS-4, 99.1% (16S rRNA)	OM189529	this study
Streptomyces sp. IC-207	*S. camonocapitis* S3-30, 100% (16S rRNA)	OM189528	this study

For antimicrobial activity experiments and volatile trapping *Streptomyces* sp. IC-207 was cultivated on soy flour mannitol medium (20 g L^{-1} soya flour (Do-It, Barneveld, The Netherlands), 20 gL^{-1} mannitol, 15 g L^{-1} agar (Kobe I, Carl Roth, Karlsruhe, Germany)), all other bacterial strains were cultivated on nutrient medium (5 g L^{-1} peptone (Bacto™ Proteose Peptone No. 3, Becton Dickinson, Franklin Lakes,

New Jersey, USA), 3 g L^{-1} beef extract (B4888, Fluka™ Analytical, Charlotte, North Carolina, USA and 07-515, Scharlau Chemie S.A., Barcelone, Spain), 5 g L^{-1} NaCl, 15 g L^{-1} agar (Kobe I, Carl Roth, Karlsruhe, Germany), pH 7.0 ± 0.2). The fungi were cultivated on potato dextrose broth (24.0 g/l potato dextrose broth (Sigma-Aldrich, Munich, Germany), 15 g/l agar (Kobe I, Carl Roth, Karlsruhe, Germany), pH 5.1 ± 0.2). All experiments were performed at 30°C. Strains that were stored at -80°C and pre-cultured for one to five days before use.

2.2. Antimicrobial Activity of Volatilomes

The antimicrobial activities of volatilomes were tested using a two-chamber Petri dish approach. In the first chamber, a mutualistic spider symbiont was incubated by preparing a cell solution (NaCl 0.9 %) of OD_{600} 0.125 which was spread using cotton swabs. *Streptomyces* sp. IC-207 and *Aureobasidium* sp. CE_32 were directly transferred from the pre-cultures using cotton swabs due to their morphology that does not allow the preparation of homogenous cell solutions. The strains were incubated for three to five days before the second chamber was incubated with one of the putative spider pathogens. *B.thuringiensis* was pre-cultivated on agar medium, diluted to 1×10^{-4} OD_{600} in NaCl (0.9 %), and 50 µL of the suspension was spread onto the second chamber. After collective incubation (24 h) the bacterial cells were scratched from the agar using a spatula and dissolved in 2 mL NaCl solution (0.9 %). The OD_{600} was measured after appropriate dilution and compared (*t*-test) to the control, which only contained the putative pathogen.

For *P. lilacinum,* a 6 mm-agar plug was cut out of the fungal pre-culture and placed face down in the second chamber. After five days of incubation photos were taken and the fungal growth area was analyzed using ImageJ (Version 1.52a, Wayne Rasband, National Institute of Health, Bethesda, Maryland, USA) and compared (*t*-test) to the control. The procedure was replicated six times.

2.3. pH Changes and Influence on Growth

To test whether the microbial volatilomes caused changes in media pH a two-chamber Petri dish approach was performed as described above (chapter 2.2.) but without the putative pathogens. After three to five days of incubation, the pH of the medium in the second chamber was measured using pH paper (Tritest L, pH 1-11, Machery-Nagel, Düren, Germany). The precision of the indicator is one pH unit. Subsequently, the putative pathogens were incubated on pH-adjusted media respectively the pH-change caused by the volatilome treatments and the growth was measured as described above (chapter 2.2.). The procedure was replicated six times.

2.4. Volatilome Trapping

The volatilomes of the five nest microbiome members were trapped. The strains were prepared as described above (chapter 2.2.) and pre-incubated for three to five days. The trapping was performed for 24 h using steel traps filled with 300 mg Tenax TA (Markes International, Llantrisant, United Kingdom) connected to special Petri dishes (based on Garbeva *et al.* 2014). Sterile media were used as controls. The procedure was replicated six times.

2.5. GC/Q-TOF Analysis

The volatiles were released from the Tenax tubes using an automated thermodesorption unit (Unity TD-100, Markes International, Llantrisant, UK) at 280°C for 8 min with a He flow of 50 mL min^{-1}. The volatiles got cold trapped at -10°C on Tenax traps and released again at 300°C within 10 min. A split ratio of 1/10 was used. The volatiles were transferred (195°C transfer line) to an Agilent 7890B GC (Agilent Technologies, Inc. Santa Clara, CA, USA) with a DB-5ms ultra inert column (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness, 122-5532 UI, Agilent Technologies, Inc. Santa Clara, CA, USA) and a run time of 35.6 min. The temperature program was set to 39°C for 1 min followed by heating up to 315°C with 10°C min^{-1} and holding for 7 min. The mass spectrometry (280°C transfer line) was performed with an Agilent 7200AB Q-TOF at 70 eV in electron ionization mode with a source temperature of 230°C. Mass spectra were recorded in full-scan-mode (m/z 30-400, 4 scans s^{-1}, 2 GHz Extended Dynamic Range). For calibration of the retention index, 1 µL alkane standard solution of C8 - C20 (40 mg L^{-1} in Hexane; 04070-5ML; Merck, Darmstadt, Germany) was spiked on a Tenax trap, flushed for 1 min to evaporate the solvent, and measured as described above.

2.6. GC/Q-TOF Data Processing

For GC/Q-TOF data processing the raw data were exported as content definition files (CDF) and imported into MZmine (Version 2.53; © Copyright 2015; Pluskal et al., 2010). Mass detection, chromatogram building (ADAP chromatogram builder), deconvolution, and peak alignment (ADAP aligner) were performed using MZmine (Version 2.53). The peak lists were exported as comma-separated values (CSV) files. The files were uploaded to MetaboAnalyst (Version 5.0, Xia Lab, Montreal, Canada; (Xia and Wishart 2011)), filtered (interquartile range), transformed (log transformation), and scaled (auto-scaling) before statistical tests were performed. Statistically significant differences between the samples and controls were identified by *t*-tests. In addition, the chromatograms were manually screened for differences between treatments and controls using MassHunter Workstation (Qualitative Analysis, Version 10.0, Agilent Technologies, Inc. Santa Clara, CA, USA). Significant differences between the samples and controls were identified likewise by *t*-tests. Compound identification was performed using AMDIS 2.72 (National Institute of Standards and Technology, USA) based on retention index comparison and mass spectrum comparison to three libraries providing more than 1.4 million spectra, namely NIST 2014 V2.20 (National Institute of Standards and Technology, Gaithersburg, Maryland, USA), Wiley 7th edition spectral libraries (Wiley, Hoboken, New Jersey, USA) and an internal library of NIOO-KNAW (Netherlands Institute of Ecology, Wageningen, The Netherlands). The retention index tolerance was ± 10 and the minimum mass spectrum match was 600 ‰. If a mass feature complied with both criteria, a visual comparison of the mass spectra was performed and designated as “tentatively identified”. The presence of five selected compounds, namely crotyl alcohol, dimethyl disulfide, 2,5-dimethylpyrazine, dimethyl trisulfide, and benzonitrile (1 µg mL^{-1} in MeOH; all purchased by Merck, Darmstadt, Germany), in the volatilomes was confirmed by measuring pure standard compounds.

178 **2.7. Antimicrobial Activity of pure Volatiles**

179 The antimicrobial activity of the five pure compounds crotyl alcohol, dimethyl disulfide, 2,5-
180 dimethylpyrazine, dimethyl trisulfide, and benzonitrile was tested using a two-chamber Petri dish
181 approach. In the first chamber a cotton disc (6 mm diameter) was placed and 5 µL pure compound was
182 applied. In the second chamber, one of the putative pathogens was incubated and the growth was
183 measured as described above (chapter 2.2.). Additionally, a mix of all compounds except dimethyl
184 trisulfide was tested for its activity. Finally, 1.25 µL of each compound was applied to a cotton disc
185 resulting likewise in a 5 µL total volume. The procedure was replicated six times.

186

187 **2.8. Changes in Antibiotic Sensitivity under Volatilome Exposure**

188 To test the influence on the antibiotic sensitivity of the putative bacterial pathogen a two-chamber Petri-
189 dish approach was combined with a standard agar diffusion test based on the protocol of Hudzicki
190 (Hudzicki 2009). In the first chamber, a nest microbiome member was cultured as described above
191 (chapter 2.2.). The spider pathogens were diluted to OD_{600} 0.125 in NaCl (0.9 %) and spread on the
192 second chamber using cotton swabs. Gentamicin and ampicillin discs (Sensi-Disc™, 10 U, Becton
193 Dickinson, Franklin Lakes, New Jersey, USA) were placed subsequently in the second chamber. After
194 collective incubation (24 h) the zones of inhibition were measured. The procedure was replicated six
195 times.

Results and Discussion

3.1. Nest Microbiome Member Volatilomes have Antimicrobial Effects

We tested four *S. dumicola* nest core microbiome members, namely *Massilia* sp. IC2-278, *Massilia* sp. IC2-477, *Sphingomonas* sp. IC-11, and *Aureobasidium* sp. CE_32, for their antimicrobial activity through volatiles against two putative spider pathogens (*Bacillus thuringiensis* and *Purpureocillium lilacinum*). The test strains were chosen due to the high relative abundance of these genera of up to 8% (*Massilia*), 10% (*Sphingomonas*), and 65% (*Aureobasidium*) of all bacterial and fungal genera in *S. dumicola* nests, respectively (Nazipi *et al.* 2021). In addition, we also investigated a *Streptomyces* sp. isolate (*Streptomyces* sp. IC-207), since *Streptomyces* species are known for their production of antimicrobial volatiles (Watve *et al.* 2001; Avalos *et al.* 2019). *B. thuringiensis* was selected based on a study showing the lethality after injection to the hemolymph of *S. dumicola* (Keiser *et al.* 2016). *P. lilacinum* was isolated from the surface of a *S. dumicola* spider (Nazipi *et al.* 2021) and suggested as a pathogen because *Purpureocillium* species are well known as spider pathogens (Shrestha *et al.* 2019). There are indications that especially in spider nests from relative humid regions (for example Otavi, Namibia) outbreaks of *Purpureocillium* species accumulate (Nazipi *et al.* 2021).

Both *Massilia* species significantly inhibited the growth of *B. thuringiensis* (**Fig. 1A, B**) but not of *P. lilacinum* (**Fig. 1F, G**). The antibacterial activity of the *Massilia* species was recently described against two Gram-negative pathogens via soluble compounds (Dahal, Chaudhary and Kim 2021). Another study found numerous biosynthetic gene clusters in *Massilia* species suggesting them as a source for novel antimicrobial compounds (Miess *et al.* 2020). The investigated *Sphingomonas* sp. IC-11 did not show any antimicrobial effects (**Fig. 1C, H**), even though some studies report antibacterial effects of other *Sphingomonas* species (Romanenko *et al.* 2007). *Streptomyces* sp. IC-207 showed only antifungal effects (**Fig. 1I**), which is in line with other studies, even though *Streptomyes* species were also shown for their antibacterial activities (Mohamed *et al.* 2021). The fungal nest member *Aureobasidium* sp. CE_32 showed both, antibacterial and antifungal activities (**Fig. 1E, J**). Diverse *Aureobasidium* species are described to produce antimicrobial volatiles. For example, *A. pullulans* was shown to inhibit the growth of several fungal fruit pathogens (Di Francesco *et al.* 2015).

Exposure to certain volatiles is known to change the pH of media (Létoffé *et al.* 2014; Avalos *et al.* 2019). Therefore, we also tested the volatilomes' effect on the media pH, which revealed that all bacterial members increased the pH by one or two units (**Fig. S1**). We grew the spider pathogens on pH-adjusted media to exclude the possibility that inhibited growth was due to the increased pH. The growth of *B. thuringiensis* was actually increased at a higher pH, while the growth of *Aureobasidium* sp. CE_32 remained unchanged, and thus excludes the change in pH as growth-inhibiting factor (**Fig. S2**). Overall, our results clearly show the antimicrobial potential of *S. dumicola* nest members' volatilomes against putative spider pathogens.

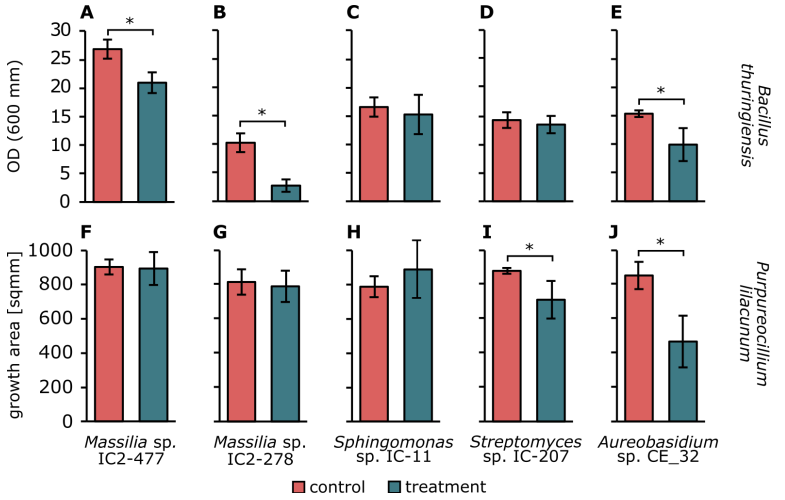

Figure 1. Antimicrobial activity of the volatilomes of the nest microbiome members *Massilia* sp. IC2-477, *Massilia* sp. IC2-278, *Sphingomonas* sp. IC-11, *Streptomyces* sp. IC-207, and *Aureobasidium* sp. CE_32 against the putative spider pathogens *Bacillus thuringiensis* (A-E) and *Purpureocillium lilacinum* (F-J). The error bars show the standard deviations. The * indicate significant differences ($p \leq 0.05$); *t*-test; $n = 6$; OD = optical density.

3.2. Nest Microbiome Members produce Antimicrobial Volatiles

The volatilome analyses of the five *S. dumicola* nest microbiome members resulted in the detection of 78 volatiles, whereof 32 could be tentatively identified based on mass spectrum and retention index comparison (**Tab. S1**). The presence of five volatiles (crotyl alcohol, dimethyl disulfide, 2,5-diemthylpyrazine, dimethyl trisulfide, and benzonitrile) was confirmed (**Fig. S3**). Most strains had unique volatilomes (**Fig. 2**). Only the volatilome of *Massilia* sp. IC2-278 overlapped for approximately two-thirds with those of *Massilia* sp. IC2-477 indicating their relatedness. Interestingly, even when *Massilia* sp. IC2-477 produced three times more volatiles than *Massilia* sp. IC2-278 both strains were able to significantly reduce the growth of *B. thuringiens* indicating that the number of volatiles cannot predict bioactivity (**Fig. 1A, B**). Supporting that observation, *Aureobasidium* sp. CE_32 produced the fewest volatiles but was the only strain with both antibacterial and antifungal activities (**Fig. 1E, J**). *Streptomyces* sp. IC-207 produced most volatiles. Indeed, *Streptomyces* species are well known for producing numerous volatiles including antimicrobial ones (Citron *et al.* 2015).

Many of the nest microbiome member volatiles were already described as microbial volatiles and/or as antimicrobials (**Tab. S1**). For example, we found dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) in all investigated bacterial volatilomes but not in the volatilome of *Aureobasidium* sp. CE_32. Indeed, these volatiles are typical bacterial sulfur-containing compounds (Weisskopf, Schulz and Garbeva 2021). Furthermore, we could show that DMDS had no antibacterial but a slight antifungal effect, whereas DMTS inhibited the growth of both pathogens completely (**Fig. 3**). Other studies showed similar antimicrobial effects of DMDS and DMTS (Kyung and Fleming 1997; Tyc *et al.* 2015). We found

2,5-dimethylpyrazine in the volatilome of *Massilia* sp. IC2-477 which showed antibacterial but no antifungal effect as volatile (**Fig. 3**). Interestingly, next to the antibacterial activities of pyrazines (Dhouibi *et al.* 2020) other studies also report antifungal activities induced by 2,5-dimethylpyrazine (Vlassi *et al.* 2020). Furthermore, we tested crotyl alcohol and benzonitrile showing antibacterial and antifungal and only antifungal activities respectively (**Fig. 3**). Finally, we also tested a mixture containing all tested volatiles (except for DMTS due to its strong antimicrobial effect) because the combination of volatiles may change their activity compared to single volatiles (Tyc *et al.* 2015). Indeed, the antibacterial activity of the volatile blend was increased compared to the single volatiles except crotyl alcohol, even though the antifungal activity was not changed. However, the complete volatilome inside a spider nest would be more complex and possibly include compounds that are emitted only as the result of microbial interactions. Even when the investigated strains, except *Streptomyces*, are members of the nest core microbiome they are part of a diverse microbiome (Nazipi *et al.* 2021).

Interestingly, some compounds we found in the nest microbiome member volatilomes were shown in other (social arthropod) ecosystems. Isoprenol, produced by *Streptomyces* sp. IC-207 in this study, was also found in propolis (“bee glue”) which is known for its antimicrobial activities (Kaskoniene *et al.* 2014; Almuhayawi 2020). It was also reported to be produced by *Pseudomonas yamanorum*, a symbiont from bats potentially protecting them from *Pseudogymnoascus destructans*, which causes white-nose syndrome (Li *et al.* 2021). Interestingly, some nest microbiome members produced volatiles that were also reported in secretes of social insects. For example, 2-Methyl-1-butanol, produced by *Aureobasidium* sp. CE_32 in our study, was previously found in termite queen pheromones, where the compound is believed to aid in egg protection due to its antifungal activity (Matsuura and Matsunaga 2015). Likewise, 2-heptanone, produced by *Aureobasidium* sp. CE_32, was reported as the major component of the gland secret of leaf-cutting ants and showed both antibacterial and antifungal activities (Mendonca *et al.* 2009).

In a previous study, we analyzed the *in situ* volatilomes of *S. dumicola* nests (Lammers *et al.* 2021). We found six volatiles with the same retention indices in both, the *in vitro* nest member volatilomes of the present study (**Tab. S1**) and the *in situ* study. In detail, volatiles with retention indices of 890, 1200, 1227, 1243, 1534, and 1561 are present in both data sets. However, we could not identify these volatiles in both the *in vitro* isolate volatilomes and the *in situ* nest volatilomes and a comparison only based on the retention indices would be highly speculative. Indeed, we found two dimethylundecanes (4,4- and 2,6-) produced by *Sphingomonas* sp. IC-11 and two different ones (3,5- and 4,6-) in the *in situ* volatilomes but these alkanes are not known for any bioactivity yet. Even though we could not find the same volatiles in both studies we could identify volatiles with antimicrobial potential produced by the nest (core) microbiome members indicating their contribution to pathogen protection. The *in situ* volatilome in a spider nest is in all probability way more complex compared to those of isolated microorganisms because it most likely includes next to numerous bacterial and fungal volatiles also volatiles from surrounding plants, the spiders themselves, their (dead) prey, etc. Whereas in a spider nest under *in situ* conditions were up to 83 volatiles identified (Lammers *et al.* 2021) we could only identifiy between 10 and 36 in the isolate’s volatilomes (**Tab. S1**). Furthermore, the volatilomes produced by (micro)organisms might be influenced by the presence of other (micro)organisms as

292 discussed above (Tyc *et al.* 2015) and the antimicrobial activity can be different when combining several
293 volatiles as shown in this study (**Fig. 3A**).

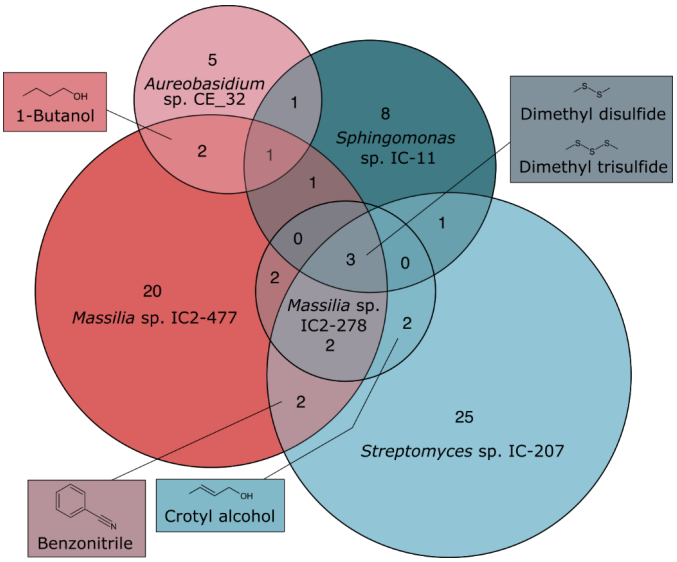

Figure 2. Volatilome comparison of the nest microbiome members *Massilia* sp. IC2-477, *Massilia* sp. IC2-278, *Sphingomonas* sp. IC-11, *Streptomyces* sp. IC-207, and *Aureobasidium* sp. CE_32 by an Euler plot. The plot is based on all identified and unknown volatiles. The areas are proportional to the number of volatiles. The boxes show those volatiles that were produced by several members and could be identified. Notice that Euler plots only describe data by approximation; for details see Tab. S1.

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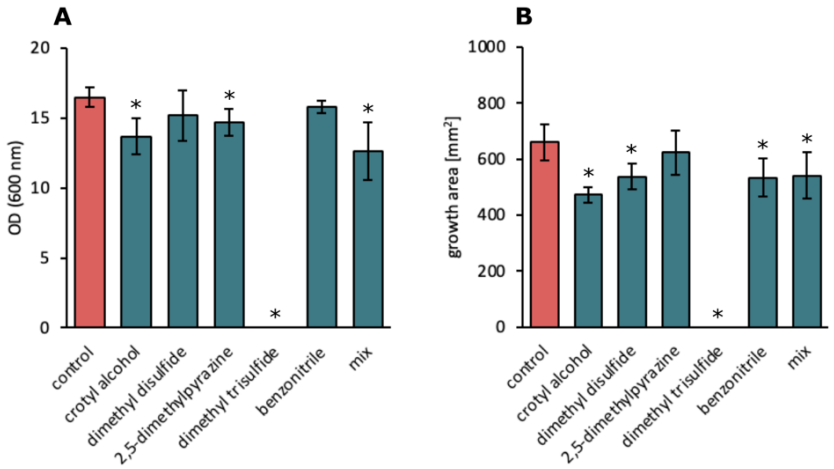

Figure 3. Influence of pure volatiles found in the volatilomes of the nest microbiome members on *Bacillus thuringiensis* (A) and *Purpureocillium lilacinum* (B). The error bars show the standard deviations. The * indicate significant differences ($p \leq 0.05$); *t*-test; $n = 6$; OD = optical density; mix = mix of all compounds except dimethyl trisulfide.

295

296 **3.3. Nest Microbiome Member Volatilomes influence Antibiotic Sensitivity**

297 Volatile and soluble compounds are often produced at the same time and can have synergistic or
298 additive effects (Tyc *et al.* 2017). Furthermore, volatiles can modulate microbial sensitivity to antibiotics
299 (Létoffé *et al.* 2014; Schmidt *et al.* 2015; Avalos *et al.* 2019). In this study, the sensitivity of *B.*
300 *thuringiensis* against the conventional antibiotics gentamicin and ampicillin was tested in presence of
301 the volatilomes of the nest microbiome members. The sensitivity of *B. thuringiensis* against gentamicin
302 was increased when exposed to the volatilomes of *Massilia* sp. IC2-278, *Sphingomonas* sp. IC-11, and
303 *Streptomyces* sp. IC-207, but decreased when exposed to the volatilome of *Aureobasidium* sp. CE_32
304 (**Fig. 4**). The sensitivity against ampicillin was only influenced when exposed to the *Aureobasidium* sp.
305 CE_32 volatilome. Due to those findings, volatiles were often discussed to counter the antimicrobial
306 resistance crisis by supporting and/or replacing old-school antibiotics (Avalos *et al.* 2018). Our results
307 highlight the potential of volatiles to combat pathogens and to complement conventional antibiotics.

308

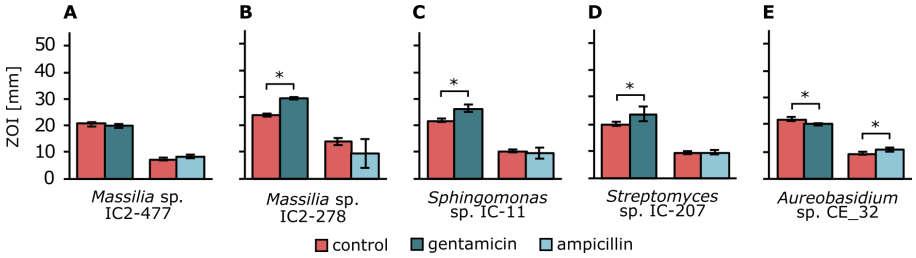

Figure 4. Influence of the volatilomes of the nest microbiome members *Massilia* sp. IC2-477, *Massilia* sp. IC2-278, *Sphingomonas* sp. IC-11, *Streptomyces* sp. IC-207, and *Aureobasidium* sp. CE_32 on the sensitivity of the putative spider pathogen *Bacillus thuringiensis* against gentamicin and ampicillin. The error bars show the standard deviations. The * indicate significant differences ($p \leq 0.05$); *t*-test; $n = 6$; ZOI = zone of inhibition.

309

310 **3.4. Conclusion**

311 Our study shows that *Stegodyphus dumicola* nest microbiome members emit different blends of
312 volatiles with diverse antimicrobial activities against the putative spider pathogens *B. thuringiensis* and
313 *P. lilacinum*. The large discrepancy between the *in vitro* isolate volatilomes and the *in situ* nest
314 volatilomes indicates the need for further research to understand the role of microbial interactions for
315 volatile production and the importance of volatiles in host-symbiont interactions. Finally, microbial
316 volatilomes may offer novel approaches to counter antibiotic resistance.

317

Supplementary Materials:

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Conflicts of Interest: The authors declare no conflict of interest.

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411

Antimicrobial Volatiles emitted by Members of the Nest Microbiome of Social Spiders (Supplementary Material)

Raw Data

The GC/Q-TOF raw data can be found under the link: https://doi.org/10.5281/zenodo.5789423
For technical reasons, some files are named after the best BLAST match (**Tab. 1**).

Figures and Tables

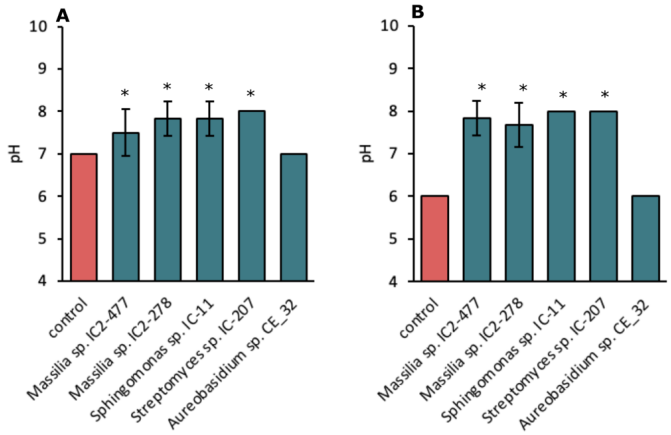

Figure S1. Influence of the volatilomes of the nest microbiome members *Massilia* sp. IC2-477, *Massilia* sp. IC2-278, *Sphingomonas* sp. IC-11, *Streptomyces* sp. IC-207, and *Aureobasidium* sp. CE_32 on the pH of nutrient broth medium (A) and potato dextrose medium (B). The error bars show the standard deviations. The * indicate significant differences ($p \leq 0.05$); *t*-test; $n = 6$.

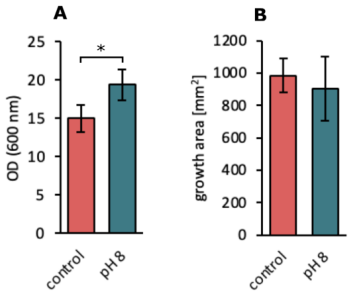

Figure S2. Influence of media with increased pHs on the growth of *Bacillus thuringiensis* on nutrient broth medium (A) and *Purpureocillium lilacinum* on potao dextrose medium (B). The pH of the control media were 7 (nutrient broth medium) and 6 (potato dextrose medium). The error bars show the standard deviations. The * indicate significant differences ($p \leq 0.05$); *t*-test; $n = 6$; OD = optical density.

Table S1. List of (tentatively) identified compounds in the volatilomes of the (core) nest microbiome members *Massilia* sp. IC2-477 (A), *Massilia* sp. IC2-278 (B), *Sphingomonas* sp. IC-11 (C), *Streptomyces* sp. IC-207 (D), and *Aureobasidium* sp. CE_32 (E). Detection of a volatile is indicated by ×; *n* = 6; The bioactivity was shown in this study and/or by other studies against bacteria (b), fungi (f), insects (i), nematodes (n), oomycetes (o), tumors (t), or viruses (v). Five compunds were confirmed by comparing to spectra of pure compunds ([C]).

RI	Compound Name	Formular	A	B	C	D	E	Bioactivity	Reference
558	unknown		×						
582	unknown		×	×	×	×			
592	unknown		×			×	×		
600	unknown		×	×					
607	unknown				×		×		
618	unknown				×				
625	2,3-Dimethyl-2-butene	C_6H_{12}				×			
627	unknown		×				×		
638	unknown		×						
639	Crotyl alcohol[C]	C_4H_8O		×	×			b, f	this study
640	2-Isopropyloxetane	$C_6H_{12}O$			×				
641	unknown					×	×		
649	unknown		×	×	×				
671	unknown		×						
674	1-Butanol	$C_4H_{10}O$	×				×	b, f	(Joo, Kim and Lee 2010; Chaves-López *et al.* 2015; Lim *et al.* 2017; Mu *et al.* 2017; Calvo *et al.* 2020)
698	unknown		×		×				
706	unknown		×						
708	S-Methyl thioacetate	C_3H_6OS			×			f	(Ossowicki, Jafra and Garbeva 2017; Dausset *et al.* 2020)
716	unknown					×			
719	Methyl isothiocyanate	C_2H_3NS	×					b, f	(Lin *et al.* 2000; Duque *et al.* 2001; Pawlik, McFall and Zea 2002; Li *et al.* 2013)

734	unknown						×		
738	Isoprenol	$C_5H_{10}O$			×			f	(Li *et al.* 2021)
739	unknown						×		
742	2-Methyl-1-butanol	$C_5H_{12}O$					×	f, o	(Batista Fialho *et al.* 2011; Fialho *et al.* 2011; Matsuura and Matsunaga 2015; Sanchez-Ortiz *et al.* 2016)
750	Dimethyl disulfide[C]	$C_2H_6S_2$	×	×	×	×		b, f, n, o	this study, (Kyung and Fleming 1997; Bendimerad *et al.* 2005; Li *et al.* 2010; Zhang, Mallik and Zeng 2013; Sheoran *et al.* 2015; Tyc *et al.* 2015; Zuo *et al.* 2015; Ossowicki, Jafra and Garbeva 2017; Mannaa and Kim 2018; Rojas-Solis *et al.* 2018)
762	unknown				×				
779	Methyl 2-methylbutyrate	$C_6H_{12}O_2$			×			b, f	(Bail *et al.* 2009; Lawal *et al.* 2018; Lyu *et al.* 2020)
795	unknown				×				
797	Cyclopentanone	C_5H_8O	×						
810	1,3-Dithietane	$C_2H_4S_2$			×				
811	unknown		×						
852	S-Methyl 2-methylpropanethioate	$C_5H_{10}OS$			×				
853	unknown		×						
867	Methyl tiglate	$C_6H_{10}O_2$			×				
884	unknown				×				
889	2-Heptanone	$C_7H_{14}O$					×	b, f	(Mendonca *et al.* 2009; Lee *et al.* 2017)
890	unknown		×	×	×				
897	unknown			×					
915	unknown			×					
916	2,5-Dimethylpyrazine[C]	$C_6H_8N_2$	×					b, f, o, t	this study, (Wang and Tao 2009; Dhouibi *et al.* 2020; Vlassi *et al.* 2020)

943	S-Methyl 3-methylbutanethioate	$C_6H_{12}OS$			×				
974	Dimethyl trisulfide[C]	$C_2H_6S_3$	×	×	×	×		b, f, o	this study, (Kyung and Fleming 1997; Kim *et al.* 2004; Bendimerad *et al.* 2005; Li *et al.* 2010, 2010; Zhang, Mallik and Zeng 2013; Casiglia *et al.* 2015; Sheoran *et al.* 2015; Tyc *et al.* 2015; Ossowicki, Jafra and Garbeva 2017)
986	unknown			×	×				
988	Benzonitrile[C]	C_7H_5N	×		×			f	this study
1022	2,6-Dimethylnonane	$C_{11}H_{24}$				×			
1029	unknown		×	×					
1054	unknown					×			
1060	4-Methyldecane	$C_{11}H_{24}$				×		f	(Alijani *et al.* 2019)
1063	unknown				×				
1066	S-Methyl methanethiosulphonate	$C_2H_6O_2S_2$	×						
1108	unknown		×						
1114	Phenylethyl alcohol	$C_8H_{10}O$					×	b, f	(Baskaran *et al.* 2015)
1117	unknown				×				
1135	unknown		×			×			
1155	unknown				×				
1179	unknown				×	×			
1200	unknown				×				
1209	4,4-Dimethylundecane	$C_{13}H_{28}$				×			
1212	2,6-Dimethylundecane	$C_{13}H_{28}$				×			
1214	unknown				×				
1227	Dimethyl tetrasulfide	$C_2H_6S_4$			×			b, f	(Kim *et al.* 2004; Mastelic, Blazevic and Kosalec 2010)
1243	(1E)-1-Ethylidene-7a-methyloctahydro-1H-indene	$C_{12}H_{20}$			×				
1303	unknown		×						
1319	Phthalic anhydride	$C_8H_4O_3$			×				

1319	1,2-Benzenedicarboxylic acid	$C_8H_6O_4$		×		b, f, i	(Rao *et al.* 2010; Mushtaq *et al.* 2013; Ullah *et al.* 2014; Jangir *et al.* 2018; Rubab *et al.* 2018, 2019)
1474	unknown		×				
1476	Dimethyl pentasulfide	$C_2H_6S_5$		×			
1492	1-Pentadecene	$C_{15}H_{30}$	×			b, f	(Sheoran *et al.* 2015; Kumari, Menghani and Mithal 2019)
1534	Sulfur compound			×			
1561	unknown				×		
1673	unknown		×				
1679	unknown		×				
1686	unknown		×				
1700	2-Hexadecanol	$C_{16}H_{34}O$	×				
1757	unknown			×			
1903	unknown		×				
2012	unknown		×				
2093	Sulfur compound			×			

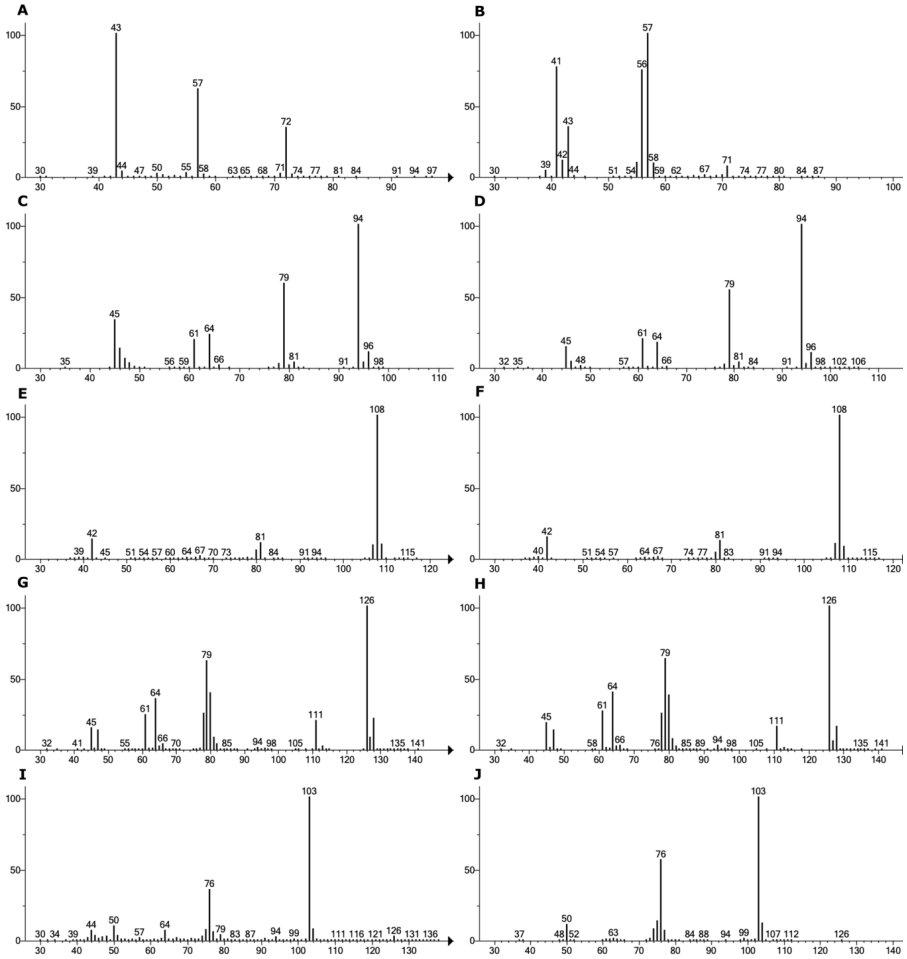

Figure S3. Mass spectra of the identified compounds in the samples (left) and from the pure compounds (right). crotyl alcohol (A, B), dimethyl disulfide (C, D), 2,5-dimethylpyrazine (E, F), dimethyl trisulfide (G, H), benzonitrile (I, J).

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FURTHER PUBLICATIONS

Unglaube, F., **Lammers, A.**, Kreyenschulte, C. R., Lalk, M., and Mejía, E. (2021). Preparation, Characterization and Antimicrobial Properties of Nanosized Silver-Containing Carbon/Silica Composites from Rice Husk Waste. ChemistryOpen 10, 1244–1250. doi:10.1002/open.202100239.

Lammers, A. (2021). 78 °N: Eine Expedition in das Arktische Ökosystem. Invited talk for the series “Familien-Universität” by the University of Greifswald; Recording: https://grypstube.uni-greifswald.de/videos/watch/da3836f1-5c7f-426d-ad22-8362f3d2f658?start=4m6s

Lammers, A. (2019). Die Spinnen, die Wissenschaftler. Von sozialen Krabbeltieren aus Namibia und Antibiotikaresistenzen. Opening talk for the 24-Hours-Lecture 2019 of the University of Greifswald

Lammers, A. (2019). Was haben Spinnen aus Namibia mit Antibiotikaforschung zu tun? Public talk for the series “Universität in der Region” by the University of Greifswald

Lammers, A., Garbeva, P., Zweers, H., Schramm, A., Lalk, M. (2019). Search for Bioactive Compounds Produced by Social Spider Holobionts. Poster presentation for project marketing at the VAAM 2019 in Jena.

“And we are not at the end of the penicillin story.”

(Fleming, 1945)

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ENGLISH

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