Development of the first database of microbial volatile organic compounds and analysis of the skin bacterial volatiles and their effect in bacteria-bacteria interactions

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1. Development of the first database of microbial volatile organic compounds and analysis of the skin bacterial volatiles and their effect in bacteria-bacteria interactions

Microorganisms are ubiquitous in our biosphere and therefore influence considerably the development and the evolution of life on earth. Likewise, they also participate to maintain homeostasis in nature. To achieve this, microorganisms are for example able to interact with their environment by producing compounds with antibiotic, antifungal, antinematicidal, plant growth modulating or semiochemical properties. Among these compounds are macro-molecules like enzymes, peptides, some antibiotics or toxins (Bastos et al., 2009; Götz et al., 2014; Fetzner, 2015). In contrast to macromolecules, there are also small molecules with low molecular weight released, which are often volatile due to their high vapour pressure and low boiling point. Consequently, they cannot only act at the site of their production but also at long distances (Schulz and Dickschat, 2007). In the recent years, these microbial volatile compounds have regained attention, focussing on the following main questions: Which volatile bouquets are emitted by microorganisms? Which impact on fitness (health), development and growth do they have on the receiving organism? Are these volatiles useful for any applications? Therefore, the study presented here was conducted to address these questions.

1.1. Establishing of the microbial volatile database (“mVOC”)

Over the past decade, an increasing number of studies have been conducted by different research groups to determine the structures, chemical natures, biological and ecological roles of volatiles produced by microorganisms (reviewed in Schulz and Dickschat, 2007; Effmert et al., 2012). A considerable number of compounds were shown to be produced by bacteria and fungi among which some were completely new to the nature (von Reuß et al., 2010; Weise et al., 2012). Nevertheless, the information on volatile emission from microorganisms were scattered in the literature. Considering the great and increasing interest of researchers regarding mVOCs, it became apparent that a comprehensive compilation of existing data on volatile emission from bacteria and fungi, which would promote and facilitate crosstalk

between scientists, was presently missing. To address this need, a mVOC database was developed for public use (Lemfack et al., 2014). It is a user-friendly compilation of microbial volatiles extracted from the literature and available online at http://bioinformatics.charite.de/mvoc/. The data were acquired by an extensive literature search in Pubmed (http://www.ncbi.nlm.nih.gov/pubmed). All the sources used are described in the database and linked to the original paper (figure 1). Up-to-date, more than 1200 compounds are filed and assigned to 490 bacterial and 135 fungal species. Furthermore, "mVOC" is the first online database containing information about mVOCs and their emitting organisms. The user interface offers several search options, for instance, by species name, Pubchem ID, structure, molecular weight and logP value (figure 1). Signature tables can also be generated by clicking on "signature"; it shows all microorganisms emitting the same compounds as the chosen species, and compounds emitted by just one species are highlighted in green. When available, the biological and ecological roles of the compounds were also described. In addition, the website features of the KEGG pathway maps (http://www.kegg.jp/) can easily be accessed though the Web service. Compounds of "mVOC" are mapped onto the metabolic pathways providing an opportunity for further analysis and interpretation. "mVOC" is also linked to NIST Chemistry WebBoock (http://webbook.nist.gov/chemistry/) to obtain retention indices, IR spectrum or mass spectrum data of compounds. Online upload (only by the authors) is possible, allowing a timely incorporation of new data sets.

mVOC database is an indispensable platform in this fast-growing research field. It will facilitate the study of microbial volatiles and help to better understand the biological and ecological roles and might be useful for applications of mVOCs.

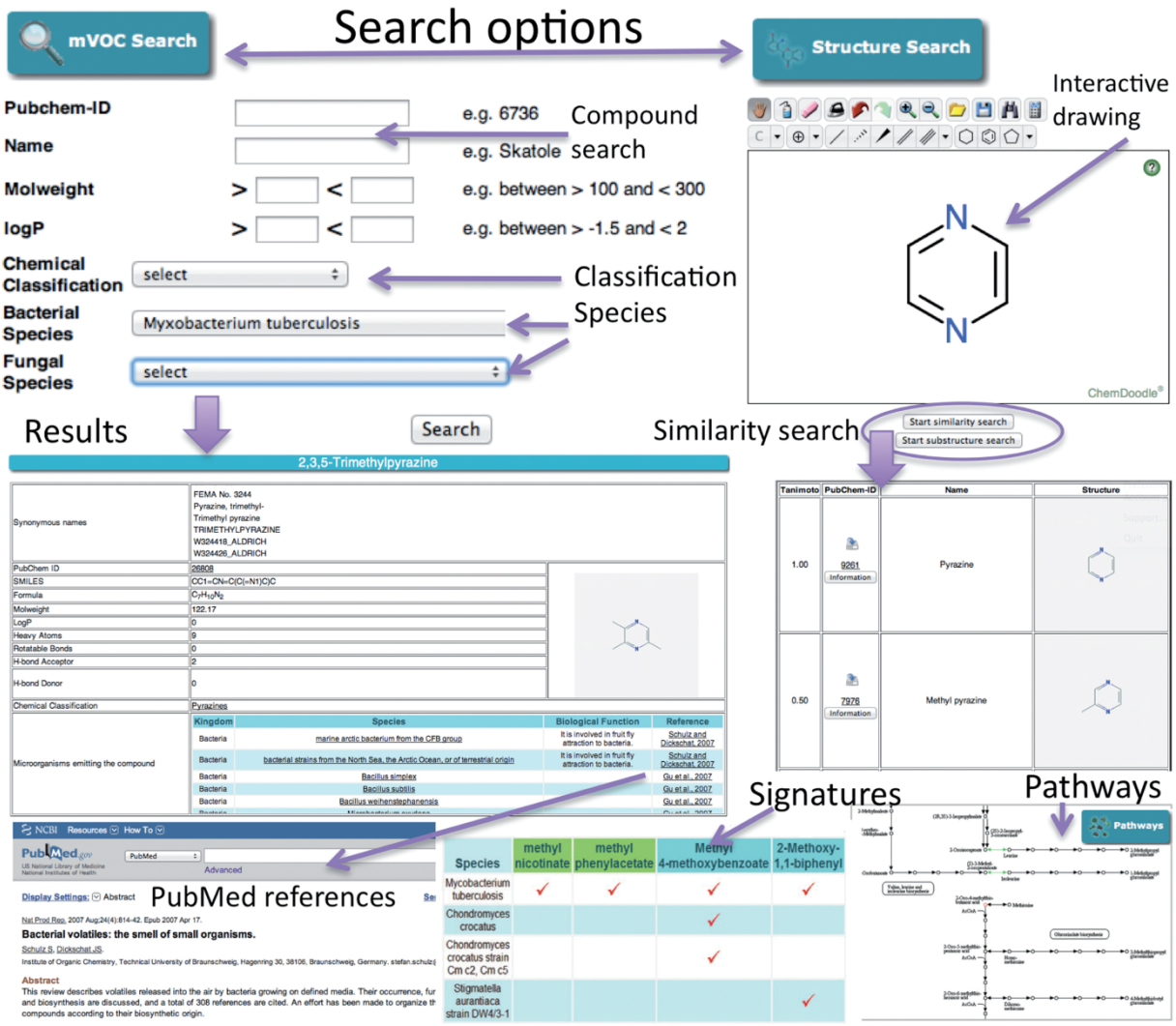

Figure 1: Overview of mVOC database.

The mVOC database offers different search options. mVOC search: a general search format for mVOCs based on PubChem ID, name of chemical compound, several molecular properties as well as the mVOC producer species. The resulting table is directly retrieved. Structure search: provides a tool for interactively drawing of a structure and performs a structure or substructure search. The result table shows volatile compounds similar to the search entry (similarity search) or volatile compounds including a substructure that is similar to the search entry (substructure search). By clicking on 'Information', one will be directed to the result table of the microbial VOC. Signatures: the signature table shows all species emitting the same compounds as the chosen species. Compounds emitted by just one species are highlighted in green. KEGG pathways: exemplarily showing parts of the 2-oxocarboxylic acid metabolism pathway.

1.2. Meta-analysis approach for assessing the diversity and specificity of mVOCs

mVOC database is an essential tool to apply meta-analysis approaches to study microbial volatile compounds. The database was used to extract the habitats of which volatile emitting microorganisms have been isolated so far. The microorganisms were mainly isolated from animals, humans, clinical sources, food products, fresh water, marine environment, plants, plant waste, rhizosphere and soil. The most listed species were isolated from plants (70), the aquatic environment (66) and the soil (61) (Piechulla and Lemfack, 2016). Meta-analysis approach revealed that habitat specificity has little or almost no influence on VOC spectra of microorganisms (Schenkel et al., 2015; Piechulla and Lemfack, 2016), confirming the results of Fiddaman and Rossall (1993) which suggested that it is not the location of isolation, but rather the nutritional supply and metabolic capabilities that are relevant for the microbial volatile profiles. Nevertheless, when correlating current literature and knowledge on plant root volatiles with belowground microbial volatile data extracted from mVOC database, Schenkel et al. (2015) showed that microorganisms belonging to the rhizosphere preferentially produce volatile signals, which are also emitted by plant roots. These volatile signals might then specifically modulate the growth and fitness of neighbouring plants. However, among the considerable number of compounds produced by bacteria and fungi described in "mVOC" database, the biological and/or ecological roles of ca. 7 % of each single volatile have been described. More often only the effects of the microbial volatile bouquets were described. It has been repeatedly shown that different microbial volatile blends influence growth, differentiation, stress response, inter/intra-species and inter-kingdom communications of diverse organisms (reviewed in Audrain et al., 2015; Hung et al., 2015; Schmidt et al., 2015). Data mining showed that bacteria might be capable of synthesizing structurally more diverse volatiles than fungi. Alcohols, ketones and carboxylic acids are the most frequently emitted chemical classes. 2-Phenylethanol, 3-methylbutan-1-ol, dimethyl disulfide and dimethyl trisulfide (in ascending order) were significantly more often released by bacteria while 1-octen-3-ol was the most frequently emitted fugal volatile (Schenkel et al., 2015; Piechulla and Lemfack, 2016). 2-Phenylethanol has a characteristic rose-like odor and it is well described as antimicrobial agent (Fraud et al., 2003; Zhu et al., 2011; Liu et al., 2014). This volatile has also repellent effects; it reduced significantly oviposition in flies (Lam et al., 2010) and attractiveness to mosquitoes (Verhulst et al., 2011; Verhulst et al., 2013).

In contrary, 3-methylbutan-1-ol is described as a good fly and mosquito attractant (Epsky et al., 1998; Verhulst et al., 2011). Moreover, Splivallo et al. (2007) showed that this mVOC has also plant growth inhibitory properties. Dimethyl disulfide and dimethyl trisulfide are prominently emitted sulfur-contain volatiles. Mainly produced by soil bacteria and to the lesser extent by fungi, these compounds, especially dimethyl disulfide, inhibit biofilm formation as well as the growth of several bacteria and fungi (Fernando et al., 2005; Dandurishvili et al., 2010; Plyuta et al., 2016). They were also described as antinematicidal and were able to influence plant growth (Gu et al., 2007; Kai et al., 2010; Meldau et al., 2013). 1-Octen-3-ol has a characteristic fungal smell (Pyysalo, 1976) and acts as antifungal or plant growth inhibitor (Chitarra et al., 2004; Splivallo et al., 2007; Berendsen et al., 2013).
All together, these few examples of mVOCs and their functions clearly indicated that mVOCs mainly affect insect behaviours as well as plant or microbial growth. They are then able to heavily influence interactions between different organisms within and across diverse ecological niches. It is also clear that one volatile can be produced by numerous organisms to a different end while some mVOCs can be produced for the same functions. However, their various ecological roles highlighted here and the fact that their emission can be specific or not suggests the existence of complex volatile-base interaction networks. Therefore, understanding the functions and specificities of mVOCs also needs to consider the context in which they are produced, e.g. the complete network of interacting organisms, concentrations, activity ratios and the persistence of the volatile signals. The bioactivity of the total blend should also be taken in consideration.

1.3. mVOCs applications

Although the number of mVOCs of which the functions are known is significantly less than the number so far described, their diverse functions and properties can be exploited in biotechnological applications in several areas as summarised in figure 2 (Piechulla and Lemfack, 2016). Numerous mVOCs have distinctive pleasing aroma, therefore they can be used in **food products** to improve the flavour of many modern fruits and vegetables. Likewise, the microbial-based volatile aromas of foodstuff such as wine, dairy products, and mushroom are continuously analysed to monitor and improve their quality (Karlshøj et al., 2007). In addition, the **perfume industry** has an increasing demand for new scents and although traditionally plants were the sources for aromas and fragrances, many other sources

including microorganisms are now coveted (Gupta et al., 2015). In contrast to well-smelling aromas, microbes can also release "off" flavour like geosmin, which is for example responsible for the muddy odor in several commercially important freshwater fish such as carp and catfish (Vallod et al., 2007). Since the repugnant smell of rotting organic matter results from the production of volatile compounds by bacteria and fungi, mVOCs are good markers for spoiled food products and crops (Casalinuovo et al., 2006). They can also be used as indicators of damp **buildings** and other hardware. Their detection provides a non-destructive way to find molds inside of buildings (Matysik et al., 2008) and in the last decade, the term "sick building syndrome" was coined to refer to a set of symptoms that are experienced by residents of poor air quality albeit the results on indoor mVOCs effects on health are controversial (Korpie et al., 2009; Polizzi et al., 2012; Bennet and Inamdar, 2015). In **agriculture**, mVOCs have high potential in influencing plant health and overcoming microbial pest. They are considered as the future eco-friendly alternatives to chemical pesticides and fertilizers for sustainable agriculture (Kanchiswamy et al., 2015). Besides considering the biological and ecological roles of mVOCs being important for the homeostasis (fitness and health) of humans, animals and plants, these compounds can also be used as non-invasive markers. Individual mVOCs as well as clusters of volatiles are useful for **phenotyping** fungi and bacteria and it has been shown that even phylogenetically closely related species of microorganisms can be differentiated (Müller et al., 2013; Peñuelas et al., 2014; Cordovez et al., 2015; Kuppusami et al., 2015). Likewise, using mVOCs as non-invasive **diagnostic tools** for microbial associated diseases is an emerging field in different medical research areas and will play a significant role in earlier detection and treatment of microbial infections (Thorn and Greenman, 2012; Sohrabi et al., 2014). Last but not the least, as the limitation of fossil fuels are foreseen and contrast steadily increasing fuel requirements, mVOCs are also regarded as the next generation **biofuel** (Rude and Schirmer, 2009; Gupta and Phulara, 2015). And since the natural microbial production rates are too low to support industrial production, metabolic engineering is widely used to improve the production (Ruffing, 2013).

Overall, the studies of microbial volatiles represent an essential route in bioprospecting, with the promise of discovering novel structures of natural products that are useful for novel applications.

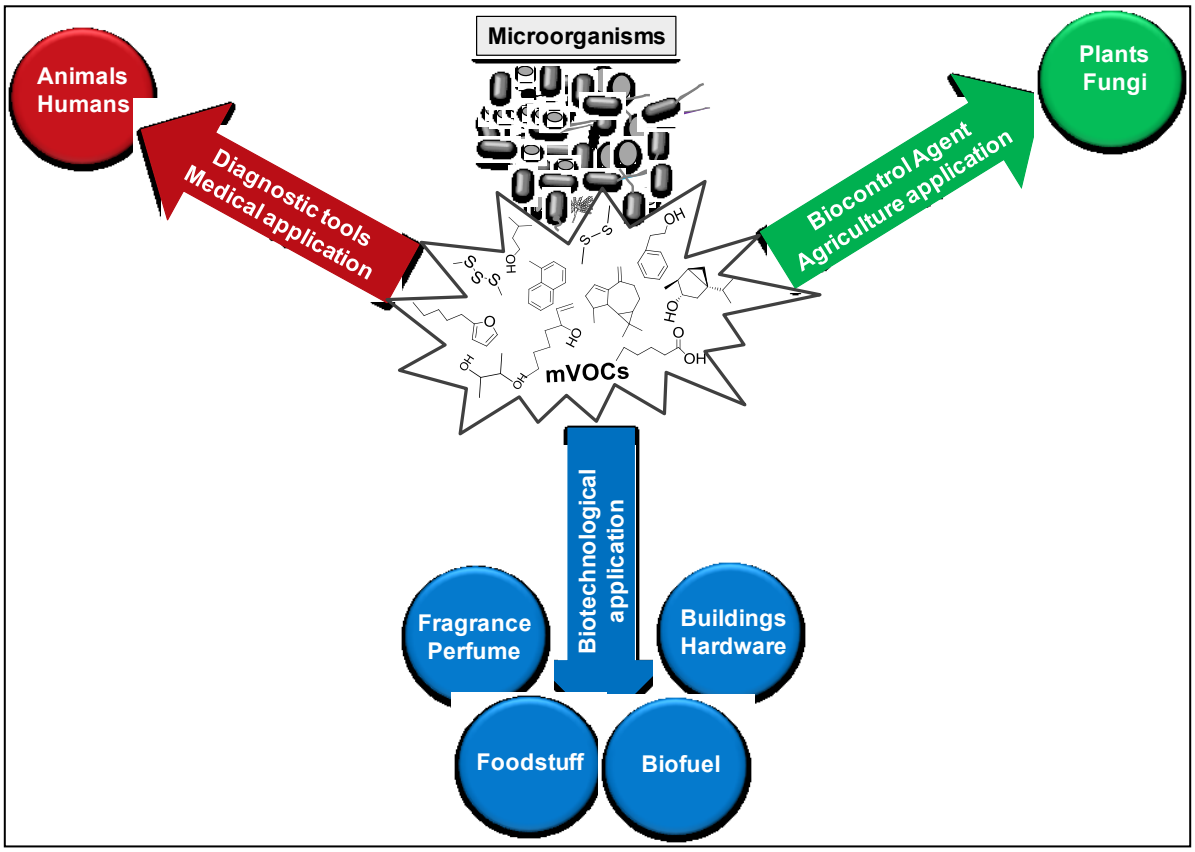

Figure 2: Overview of mVOC applications.

1.4. Analysis of the skin bacterial volatiles and their role in bacteria-bacteria interactions

By data mining, Piechulla and Lemfack (2016) showed that the most described habitats of mVOC producers are the soils/plants and aquatic environments. It is well known that there are 40 million bacterial cells in a gram of soil and a million bacterial cells in a milliliter (gram) of fresh water (Whitman et al., 1998). Furthermore, the ability of the microorganisms to produce volatiles as well as the roles of the mVOCs in mediating interactions among different organisms have been extensively study in these ecological niches. In contrast, other habitats like the human body and its diverse microbiota have been overlooked regarding volatile emissions and their potential functions, despite the fact that there are about 10^{14} bacterial cells residing in the colon (Savage, 1977) or ca. 10^{12} bacterial cells on the skin (Berg, 1996). Recent studies however proofed that microbial communities in and on the body have impact on health and disease of humans (Thorn and Greenman, 2012; Briard et al., 2016). Considering the huge number and diversity of microbes in and on the body, influential effects

of VOCs produced by microorganisms of the different microbiota (gut, oral, skin, etc.) could present a major asset in the process of discovery of new compounds for human use.

1.4.1. Skin microbiota

The skin is the body's largest organ, which serves as a physical as well as dynamic barrier between the internal and external environment. It protects the organism from harmful agents, excessive loss of water and microbial assault (Madison, 2003; Segre, 2006; Proksch et al., 2008). Since the skin is in permanent contact with the external environment, it is heavily colonized by diverse species of microorganisms, collectively known as the skin microbiota, which plays a key role in health and disease (Sanford and Gallo, 2013). The skin microbiota is made up of complex and dynamic communities of microbes including fungi, viruses and mites, but bacteria are generally dominant (Grice et al., 2009). Based on the analysis of 16S rRNA, it has been shown that around 1000 species of bacteria can be found on the human skin and they belong to 19 phyla, of which Actinobacteria, Firmicutes and Bacteriodes (mainly *Staphylococcaceae* and *Corynebacteriaceae*) predominate (Grice et al., 2009; Grice et al., 2011). Although it was recently shown that the host genome has an impact on the diversity of the skin microbiota, little is known about how its composition is controlled (Srinivas et al., 2013). Nevertheless, most skin-resident bacteria are non-pathogenic commensals and it has become apparent that some species are beneficial for their host; e.g. they are able to interact with immune cells to "educate" the skin immune system (Naik et al., 2012; Belkaid et al., 2014; Salava and Lauerma 2014). During competition for nutrients or site occupation, the skin-resident bacteria might synthesize toxic compounds to outcompete and eliminate pathogens (Schnell et al., 1988; Kellner et al., 1988; Götz et al., 2014). It is thus likely that the skin represents a habitat which is characterized by strong interactions between its normal microbial residents and/or with other environmental microorganisms. However, little is known about how these communities maintain their stabilities on the skin and their roles in health and disease. It was tempting to speculate that mVOCs might play important roles in microbial interactions and defences.

1.4.2. VOC analysis of the skin bacteria

Recently, the chemical composition of the skin odor has been analysed regarding its application in different fields like forensic studies, cosmetics or chemical ecology of host/

vector/pathogen interactions (reviewed in Dormont et al., 2013). It has been shown that the volatile profile of the skin varies greatly (the use of different sampling procedures being one of the variation factors) and the main class of compound were carboxylic acids (and derivative esters), aldehydes, alkanes, short chain alcohols and ketones (Dormont et al., 2013). To our knowledge, there were no data in the literature showing a comprehensive analysis of the VOC profiles of typical skin-resident microbes, despite the fact that the secretions produced by the skin glands are usually odourless and are subsequently transformed by bacterial metabolism into odoriferous and volatile compounds (Shelley et al., 1953; James et al., 2004; Wood and Kelly 2010; James et al., 2013; Troccaz et al., 2015). For instance, species of Corynebacteria degrade various precursor compounds found in the sweat into short branched-chain fatty acids, such as (E)-3-methyl-2-hexenoic acid, which is the primary contributor to the typical axillary odor and a key scented volatile (Zeng et al. 1991; Natsch et al., 2003). In addition, *Staphylococcus epidermidis* degrades leucine present in the sweat to produce 3-methylbutanoic acid, which is the major component of foot odor (Ara et al., 2006). Therefore, in order to gain a better understanding of the role of the skin bacterial VOCs in bacteria-bacteria interactions, the VOC profiles of different bacterial species, which are naturally found on the skin, were analysed. A comprehensive analysis of the VOC profiles of corynebacterial and staphylococcal species, the most dominant microbes on the skin, was performed. The results revealed that the VOC profiles of these bacteria differ significantly (Lemfack et al., 2016). This difference could be explained by their individual metabolisms although they were analysed under the same conditions. Kwaszewska et al. (2014) have demonstrated that corynebacterial and staphylococcal communities co-habiting on human skin do not employ the same sets of enzymatic activities to metabolise substrates like carbo-hydrates, lipids, proteins and that *Corynebacterium* spp. express less proteinase, phosphor-lipase and saccharolytic activity. These observations could also explain why the number of VOCs emitted by the *Staphyloccocus* strains studied here was fivefold higher than that of the corynebacterial strains, which allowed a clear discrimination of the members of the two genera by multivariate analysis solely based on their VOC spectra (Lemfack et al., 2016). This study highlighted that although there are common volatiles (10- and 11-methyl-2-trideca-none or 2-pentadecanone) produced by different and unrelated bacteria (see also table A1 appendix), other volatiles are strain and/or species specific. Fatty acids such as 3-methyl-butanoic and 2-methylbutanoic acid were produced by all *Staphylococcus* strains examined,

which confirmed the finding of Ara et al. (2006) and highlighted the major role of Staphylococci in foot odor formation. Contrary to the acids, 2-phenylethanol was most specific to Corynebacteria and has prompted speculation that it may be emitted by the bacteria during interspecies competition (for nutrients and/or site occupation on the skin) to eliminate other specie and/or to inhibit growth of pathogens on the skin. More interestingly, the VOC profiles of the skin bacteria, particularly those of *Staphylococcus schleiferi* isolates, were largely composed of ketones. Among these latter, there were several amino/imino ketones which were unique to the *S. schleiferi* strains and neither of these compounds has previously been reported from natural sources. The VOC profile of *S. schleiferi* DSMZ 4807 was of particular interest, as it was dominated by two compounds, 3-(phenylamino)butan-2-one and (*E*)-3-(phenylimino)butan-2-one, which were named schleiferons A and B, respectively (figure 3). Schleiferons A and B were not detectable in the bacterial culture after 24 h but were mainly produced in the stationary phase (figure 4). At 48 h, schleiferons A and B represented 34 % and 2.4 % respectively, of the total VOC spectrum, increasing to 70 % and 10 % by 72 h, and 73 % and 14 % at 96 h, respectively. Considering these remarkable VOC compounds, the effects of *S. schleiferi* DSMZ 4807 volatiles on other bacteria were further analysed.

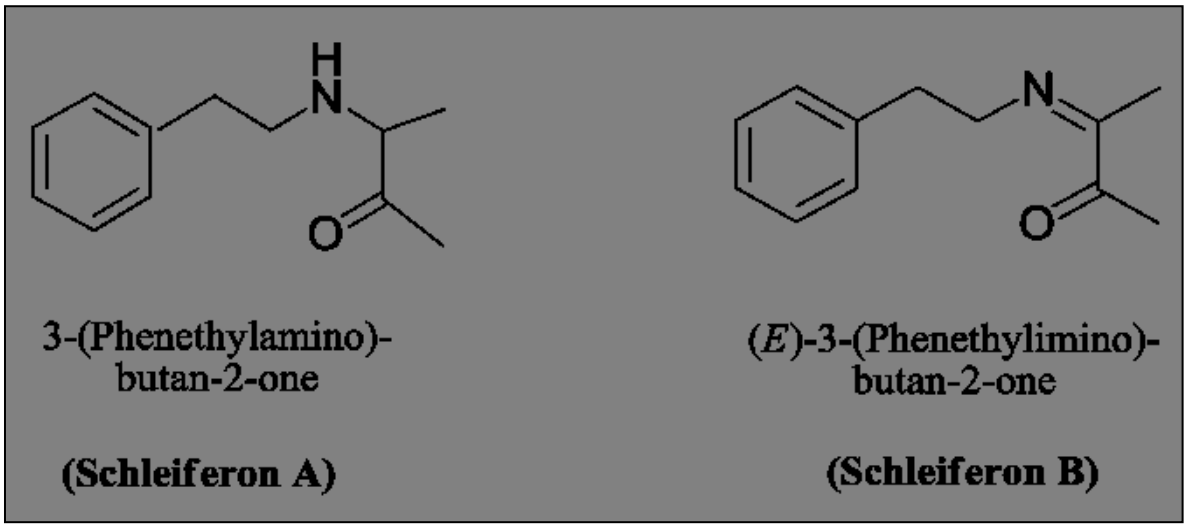

Figure 3: Structures of schleiferons A and B from *Staphylococcus schleiferi* DSMZ 4807.

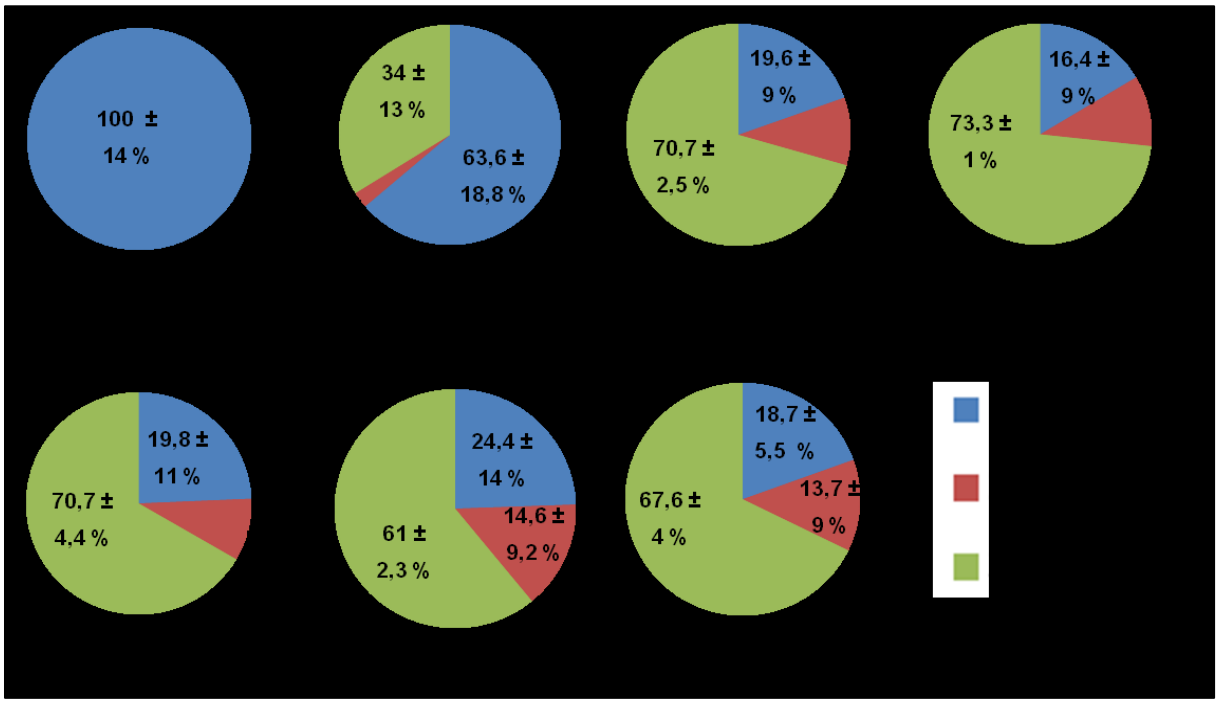

Figure 4: VOC spectrum of *Staphylococcus schleiferi* DSMZ 4807.

VOCs were quantified and the relative contributions (%) of schleiferons A and B of the VOC spectrum of *S. schleiferi* cultures were determined at intervals of 24 h over the course of 1 week (100 % (0-24 h) = 16.6 ng; 100 % (24-48 h) = 34.2 ng; 100 % (48-72 h) = 104.5 ng; 100 % (72-96 h) = 130.3 ng; 100 % (96-120 h) = 136.8 ng; 100 % (120-144 h) = 109.3 ng; 100 % (144-168 h) = 108.1 ng).

1.4.3. Effect of *Staphylococcus schleiferi* DSMZ 4807 volatiles on other bacteria

Microbe-microbe interactions are known to be mediated via secondary metabolites (Schmidt et al., 2015). To gain insight into the biological role(s) of *S. schleiferi* DSMZ 4807 volatiles, particularly schleiferons A and B, dual cultures of this bacterium with bacteria which are naturally found on the skin or with other bacteria from different ecological niches were performed. The results revealed that *S. schleiferi* volatiles selectively inhibited the growth of Gram-positive bacteria. The growth inhibition was only significant when the bacteria were co-cultivated with *S. schleiferi* cells that were in the late stationary phase, the period in which the concentrations of schleiferons A and B reached their maxima. This suggested that these compounds might contribute to the reduced growth of Gram-positive species. This notion was further supported by the finding that *Staphylococcus warneri*, which does not synthesize either schleiferon, did not affect any of the bacteria tested. Moreover, when chemically synthesised schleiferons A and B were tested separately, they both specifically inhibited the

growth of Gram-positive bacteria in a concentration-dependent manner, with schleiferon A being significantly more active than B (Lemfack et al., 2016).

During competition for nutrients or territory, it is known that skin bacteria release different antimicrobial compounds to prevent adherence of pathogens and/or competitors (Bibel et al., 1983; Cogen et al., 2010). Since schleiferons were significantly more active (3- to 16-fold) against *Corynebacterium* strains and *Micrococcus luteus* (skin bacteria) and less deleterious to *Bacillus subtilis* (rhizobacterium) and *Enterococcus* strains (gut bacteria), we speculate that these compounds are primarily directed against other Gram-positive skin bacteria. Moreover, they were also active against other skin *Staphylococcus* strains, albeit these latter were considerably less affected than Corynebacteria or *Micrococcus luteus* (table 1). Therefore, the production of schleiferons can be an advantage for Staphylococci during bacterial interactions and might help them to keep their balance on the skin. Further, among these skin bacteria, the prominent schleiferon producer *S. schleiferi* DSMZ 4807 was 2- to 8-fold more resistant to these VOCs and its growth was noticeably affected at very high concentrations, which might only be reached in the late stationary phase. Altogether, these results highlighted the role that schleiferons may have in skin bacterial interactions, suggesting that mVOCs might contribute to maintain species diversity and to shape the evolution of community composition or structure in the skin microbiome. However, the mode of action by which these compounds inhibit the growth of the Gram-positive bacteria is not yet known and remains to be investigated in the future.

In contrast to Gram-positive, growth of Gram-negative bacteria were unaffected by either *S. schleiferi* volatiles or schleiferons (e.g. *Salmonella enterica* RV4 (table 1). Surprisingly, we observed a rather drastic reduction of the red pigment prodigiosin produced e.g. by the Gram-negative bacterium *Serratia marcescens* V11649 (figure 5).

Table 1: Minimum inhibitory concentrations (MIC) of schleiferons A and B on different Gram-positive bacteria.

Bacteria		MIC (µM) of Schleiferon	
Families	Species	A	B
Corynebacteriaceae	*Corynebacterium jeikeium* V12209	34.8	281
Corynebacteriaceae	*Corynebacterium striatum* RV2	34.8	281
Corynebacteriaceae	*Corynebacterium minutissimum* ATCC 23348	69.6	563
Staphylococcaceae	*Staphylococcus schleiferi* DSMZ 4807	278	1130
Staphylococcaceae	*Staphylococcus schleiferi* H34	278	2250
Staphylococcaceae	*Staphylococcus schleiferi* V431	139	2250
Staphylococcaceae	*Staphylococcus intermedius* 9S	139	563
Staphylococcaceae	*Staphylococcus saccharolyticus* B5709	139	1130
Staphylococcaceae	*Staphylococcus sciuri* V405	139	1130
Staphylococcaceae	*Staphylococcus warneri* CCM 2730	96.7	1130
Staphylococcaceae	*Staphylococcus epidermidis* RP62A	69.6	281
Staphylococcaceae	*Staphylococcus haemolyticus* CCM 2729	69.6	563
Micrococcaceae	*Micrococcus luteus* V515	34.7	141
Enterococcaceae	*Enterococcus faecium* ATCC 51559	278	2250
Enterococcaceae	*Enterococcus faecalis* ATCC 51299	557	3380
Bacillaceae	*Bacillus subtilis* B2g	278	1130
Enterobacteriaceae	*Salmonella enterica* RV4	4450	> 10000

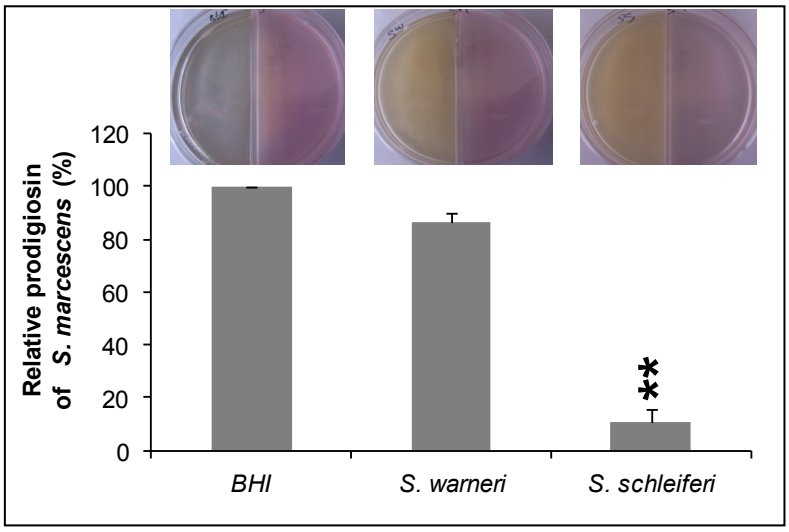

Figure 5: Effects of *Staphylococcus schleiferi* and *S. warneri* volatiles on prodigiosin production by *Serratia marcescens*.

The concentration of the pigment was determined by measuring its absorption at OD_{534} and expressed in percentage of the level produced in the BHI control culture. ** $p < 0.01$.

The name of this red pigment derives from Kraft (1902), who extracted it from *Bacillus prodigiosus*. It is a typical secondary metabolite and characteristic feature of some *Serratia* strains with antibacterial, antifungal or immunosuppressive properties (reviewed in Darshan and Manonmani, 2015). The biosynthesis of prodigiosin is controlled by a complex regulatory network involving quorum sensing system (Williamson et al., 2006). Our hypothesis was therefore that *S. schleiferi* volatiles or schleiferons inhibit quorum sensing controlled features in Gram-negative bacteria. To verify this idea, other strains were tested, *Serratia plymuthica* AS9 which produced prodigiosin and *Vibrio harveyi*, which produced bioluminescence and both were used as model organism to study quorum sensing in Gram-negative bacteria.

1.4.4. Effect of *Staphylococcus schleiferi* DSMZ 4807 volatiles on quorum-sensing-dependent phenotypes of Gram-negative bacteria

Bacterial interactions are facilitated by cell-to-cell chemical communication named quorum sensing (QS). This process is based on the production and recognition of small hormone-like signal molecules (auto-inducers), which bind to specific receptors to coordinate gene expression and regulate important bacterial phenotypes like virulence factor production, swarming motility, biofilm formation, antibiotic secretion and bioluminescence (Davies et al.,

1998; Bassler et al., 2006; Higgins et al., 2007). We found that *S. schleiferi* DSMZ 4807 volatiles inhibit quorum sensing-dependant phenotypes in all Gram-negative bacteria tested here. This inhibition was more accentuated when bacterial cultures were fumigated with *S. schleiferi* volatiles in the late stationary phase (where the production of schleiferons was maximal) (figure 6) suggesting that schleiferons might contribute to the inhibition of these phenotypes. This notion was further supported by the finding that *Staphylococcus warneri*, which does not synthesize either schleiferon, did not affected any phenotype of the bacteria tested. Moreover, when chemically synthesised, schleiferons A and B were tested separately and they both specifically inhibited the bacterial phenotypes in a concentration-dependent manner (Lemfack et al., 2016). These results were in good agreement with the literature showing that the communication system in bacteria can be disrupted by quorum sensing inhibitors known as quorum quenchers (QQ) (Defoirdt et al., 2008; Chu et al., 2013; Zhao et al., 2016). QQ molecules (enzymes and chemicals) were reviewed by Grandclément et al. (2016) and the authors have described also some QQ paradigms to exemplify the mechanisms and biological roles of QS inhibition in microbe-microbe and host-microbe interactions. QS inhibitors have diverse targets and different mode of actions; theoretically, any step of the QS pathway (synthesis, diffusion, accumulation and perception of the QS signals as well as transduction of the signal) might be affected (Defoirdt et al., 2007; Chu et al., 2013; Fetzner, 2015).

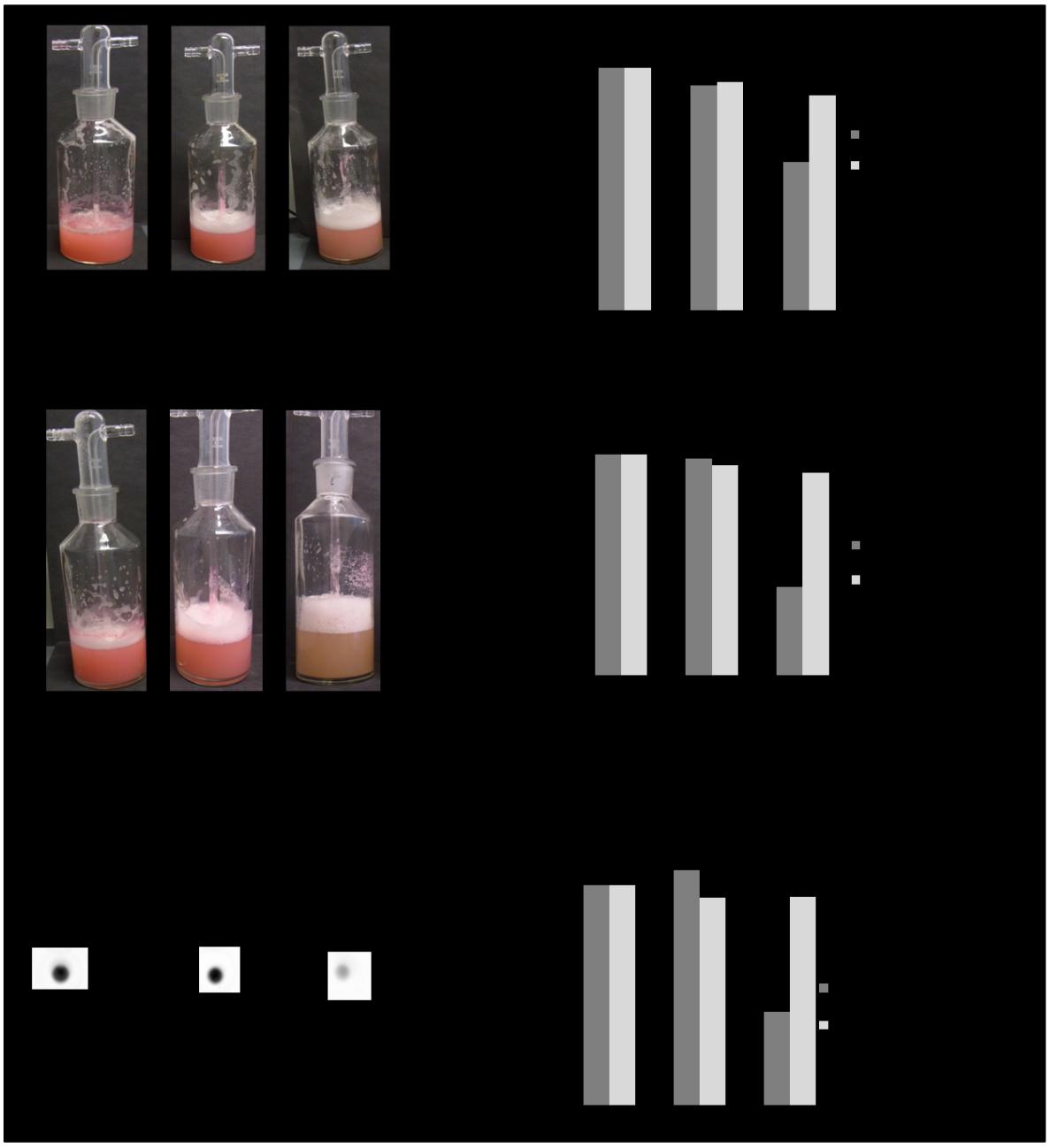

Figure 6: Volatiles of *Staphylococcus schleiferi* inhibit prodigiosin production in *Serratia marcescens* and bioluminescence in *Vibrio harveyi*.

S. marcescens was cultivated in a dual culture system for 24 h with a 48-h (**A**) or a 96-h (**B**) culture of *S. schleiferi* or *S. warneri* (BHI: brain heart infusion medium). Cell density (OD_{600} of 100 % = 4) and relative levels of prodigiosin in the culture medium (%) (OD_{534} of 100 % = 1.4) of *S. marcescens* was measured after 24 h dual cultivation of *S. marcescens* with a 48 h (**C**) or 96 h (**D**) culture of *S. schleiferi*/*S. warneri.* **E**) presents the light image of the bioluminescence of *V. harveyi* cultivated in a dual culture system for 24 h with a 96 h old culture of *S. schleiferi*/*S. warneri* and **F**) shows the relative growth (% cell density) (OD_{600} of 100 % = 5) and bioluminescence emission (%) (100% = 7.9E+05) of the *V. harveyi*. The culture medium was used as control. Data are the means of 3-5 independent experiments and bars indicate mean standard deviation. ** $p < 0.01$, *** $p < 0.001$.

1.4.5. Action mode of schleiferons in Gram-negative bacteria

In the marine bacterium *Vibrio harveyi,* induction of bioluminescence depends on a complex QS signalling cascade. At low cell density, in the absence of autoinducers, the three hybrid histidine kinases LuxN, LuxQ (in interplay with LuxP) and CqsS autophosphorylate, and transfer the phosphoryl group via a phosphorelay to the histidine phosphotransferase protein (HPr) LuxU, and subsequently to the response regulator LuxO. Phosphorylated LuxO activates transcription of five regulatory sRNAs which, together with the RNA chaperone Hfq, destabilize the transcript coding for the master regulator LuxR (Waters et al., 2005) (figure 7). When the concentration of LuxR in cells is low, induction of bioluminescence is impossible. At high cell density, in the presence of high concentrations of autoinducers, autophosphorylation is inhibited, LuxR is synthesised, and the bioluminescence phenotype is expressed (figure 7). We were therefore wondering whether schleiferons act as auto-inducer antagonists by interfering with auto-inducer receptors to influence bioluminescence production through a stimulation of the autophosphorylation cascade. As result, we found no increase in phosphorylation of the quorum sensing receptors after schleiferon A or B addition, suggesting a target(s) downstream of the hybrid histidine kinases. These results were corroborated by the schleiferon-mediated inhibition of bioluminescence in a *luxO* deletion mutant, which constitutively produces bioluminescence independently of auto-inducers and the QS receptors (Lemfack et al., 2016). It was therefore conceivable that schleiferons act downstream of the QS cascade and these other protein targets have to be investigated in the future.

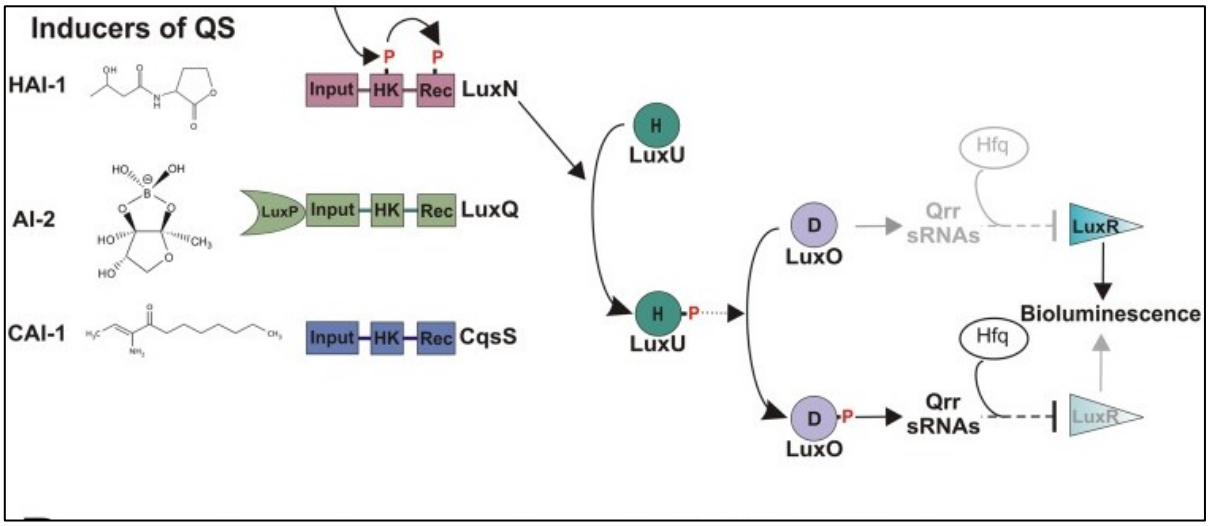

Figure 7: Schematic representation of the QS phosphorelay in *V. harveyi* (modified from Chu et al. 2013)**.** In the absence of autoinducers (HAI-1, AI-2 and CAI-1) at low cell density, each of the three receptors, LuxN, LuxQ and CqsS, respectively, are autophosphorylated at a conserved histidine by their histidine kinase domain (HK). The phosphoryl group is first transferred to the receiver domain (Rec) of the receptor kinase and then to the HPt protein LuxU. LuxP is a periplasmic binding protein. P denotes phosphorylation sites. Upon perception of the autoinducers at high cell density, autophosphorylation of the receptors and the subsequent phosphorylation cascade is inhibited.

Similarly to the bioluminescence emission of *Vibrio harveyi*, the biosynthesis of prodigiosin in *Serratia* strains is controlled by complex cascades involving quorum sensing (Williamson et al., 2006). The biosynthesis and the regulation of this red pigment has been well characterised in *Serratia* sp. ATCC 39006 (Fineran et al., 2005; Williamson et al., 2005 and 2006; Gristwood et al., 2011) and homologous genes are also found in *S. plymuthica* AS9 (KEGG SSDB database). The *pig* cluster contains 15 (*pigA-O*) genes which are co-transcribed from a promoter upstream of *pigA***.** It encodes the enzymes that catalyse the synthesis of prodigiosin, involving two different pathways for the production of the monopyrrole, 2-methyl-3-n-amyl-pyrrole and the bipyrrole, 4-methoxy-2,2'-bypyrrole-5-carbaldehyde which are then coupled in the final condensation step to form prodigiosin (Slater et al., 2003; Williamson et al., 2005). The *pig* cluster of *S. plymuthica* AS9 was shown to be transcribed both in the presence and absence of schleiferons. However, Northern blot analysis revealed that the transcriptional levels of these genes were very low in *S. plymuthica* AS9 treated with schleiferon B, which ultimately leads to the reduction of prodigiosin synthesis and accumulation observed in the bacterial culture (Lemfack et al., 2016). Further, a complex hierarchical network of regulatory proteins controls the expression of the *pig* cluster.

Fineran et al. (2005) have previously identified the PigP protein as a master transcriptional regulator of secondary metabolism in some *Enterobacteriaceae*. They found that this protein could control prodigiosin production in *S. sp*. ATCC 39006 either by directly regulating the expression of the *pig* biosynthesis cluster or indirectly via transcriptional control of genes for six other regulators ((*pigQ*, *pigR*, *rap* which overlap the quorum-sensing circuit), *pigV*, *pigS* or *pigX*). When schleiferon was added to *S. plymuthica* AS9 culture, the transcriptional level of *pigP* was significantly increased in comparison to the control, suggesting a control via this *pig* regulator. Moreover, when we analysed the transcription levels of *pigS*, we also found a significant increase of its transcription level. And *pigS* encodes for an ArsR family regulator which is able to repress the expression of other proteins (BlhA; OrfY; PmpA, B and C) involved in prodigiosin biosynthesis (Gristwood et al., 2011). In summary, the transcription level of *pigS* correlated with the activation of the expression of the master regulator PigP, and both up-regulations might favor a reduction of prodigiosin synthesis in *S. plymuthica* AS9 (Lemfack et al., 2016). In contrast to PigP, SmaR in *S. sp*. ATCC 39006 (*Lux*R homolog in *S. plymuthica* AS9) is a quorum-sensing master transcriptional repressor (Slater et al., 2003; Fineran et al., 2005). LuxR represses expression of the *pig* biosynthesis gene cluster directly or indirectly via the repression of other transcriptional regulators like PigR, PigQ or Rap (Fineran et al., 2005). When we analysed the transcription levels of *luxR* in *S. plymuthica* AS9 after application of schleiferon, we surprisingly found that they were almost unaffected. Nevertheless, the transcriptional regulators which are under the control of both quorum-dependent and –independent mechanisms (pigQ, pigR, rap) have to be investigated to completely elucidate the mode of action of schleiferon on prodigiosin production in *S. plymuthica* AS9.

Considering the increasing problem of antibiotic resistance, the use of QQ compounds and enzymes represent a promising alternative as novel anti-infective therapy tool, which can be used in different fields such as in the medical and agricultural area. Elucidation of the mode of action of schleiferons will help to determine whether they are relevant candidates for anti-infective and anti-virulence therapy studies.

1.5. Biosynthesis of schleiferons A and B

Since schleiferons A and B have never been reported to by produce by any organism, the investigation of their biosynthetic pathway was initiated here (Ravella, Lemfack, Piechulla, Schulz, in preparation). A pathway was proposed by Stefan Schulz (University of Braunschweig) (figure 8) including an unusual Voigt-Ammadori rearrangement and spontaneous oxydation of schleiferon A to B.

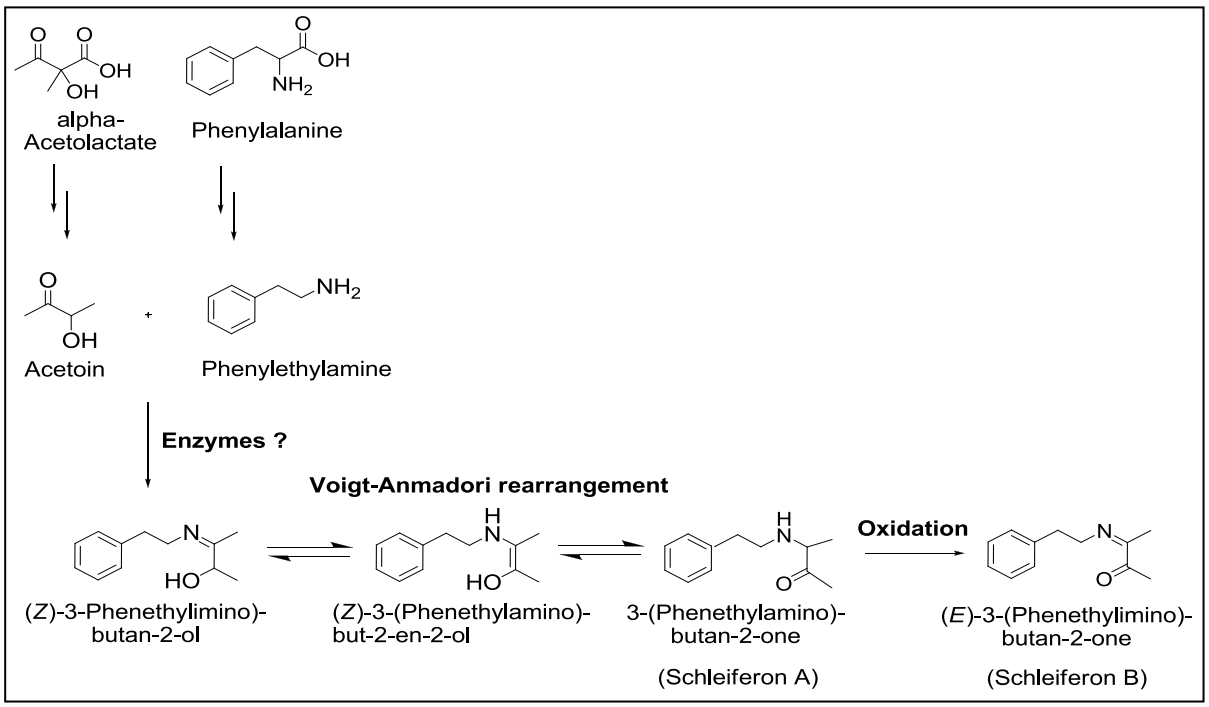

Figure 8: Proposed biosynthetic pathway of schleiferon A and B of *Staphylococcus schleiferi* DSMZ 4807.

In vitro biosynthesis was tested using the proposed substrates (acetoin and 2-phenylethylamine) and the crude extract of the bacterial culture. The results indicated that acetoin and 2-phenylethylamine are able to react and form schleiferons (figure 9). Moreover, both substrates are also found in the volatile profile of schleiferon producers (Lemfack et al., 2016). Acetoin is an essential metabolite produced by many bacteria and serves as precursor in the biosynthesis of branched-chain amino acids. Mainly formed by decarboxylation of alpha-acetolactate, it can also be secreted as by-product from pyruvate oxidation or decarboxylation reactions (reviewed in Xiao et al., 2007). Instances of bacterial production of 2-phenylethylamine are rarely found in the literature and the capacity to synthesise this compound is also not widely distributed even among Staphylococci (Landeta et al., 2007;

Bermúdez et al., 2012; Stavropoulou et al., 2015). Nevertheless, some Enterococci, as well as lactic acid bacteria isolated from food products, have been shown to synthesise this compound via decarboxylation of L-phenylalanine by tyrosine decarboxylase (Latorre-Moratalla et al., 2010; Marcobal et al., 2011; Pleva et al., 2012; Bargossi et al., 2015). It was thus speculated that 2-phenylethylamine is synthesised by *S. schleiferi* by the same mechanism or via an as yet undescribed specific L-phenylalanine decarboxylase.

The *in vitro* results did not show increased accumulation of scheiferons within time (although the substrates were still in the reaction tube). And the formation of schleiferons in absence of the crude extract (lysis buffer or boiled crude extract) in the reaction tube was also observed (figure 9), suggesting then a non-enzymatic reaction but rather, a spontaneous condensation of the two substrates. However, since schleiferon A is produced in very high amounts by *S. schleiferi*, it is possible that *in vivo*, this reaction is accelerated by metal ions, different temperature as well as pH.

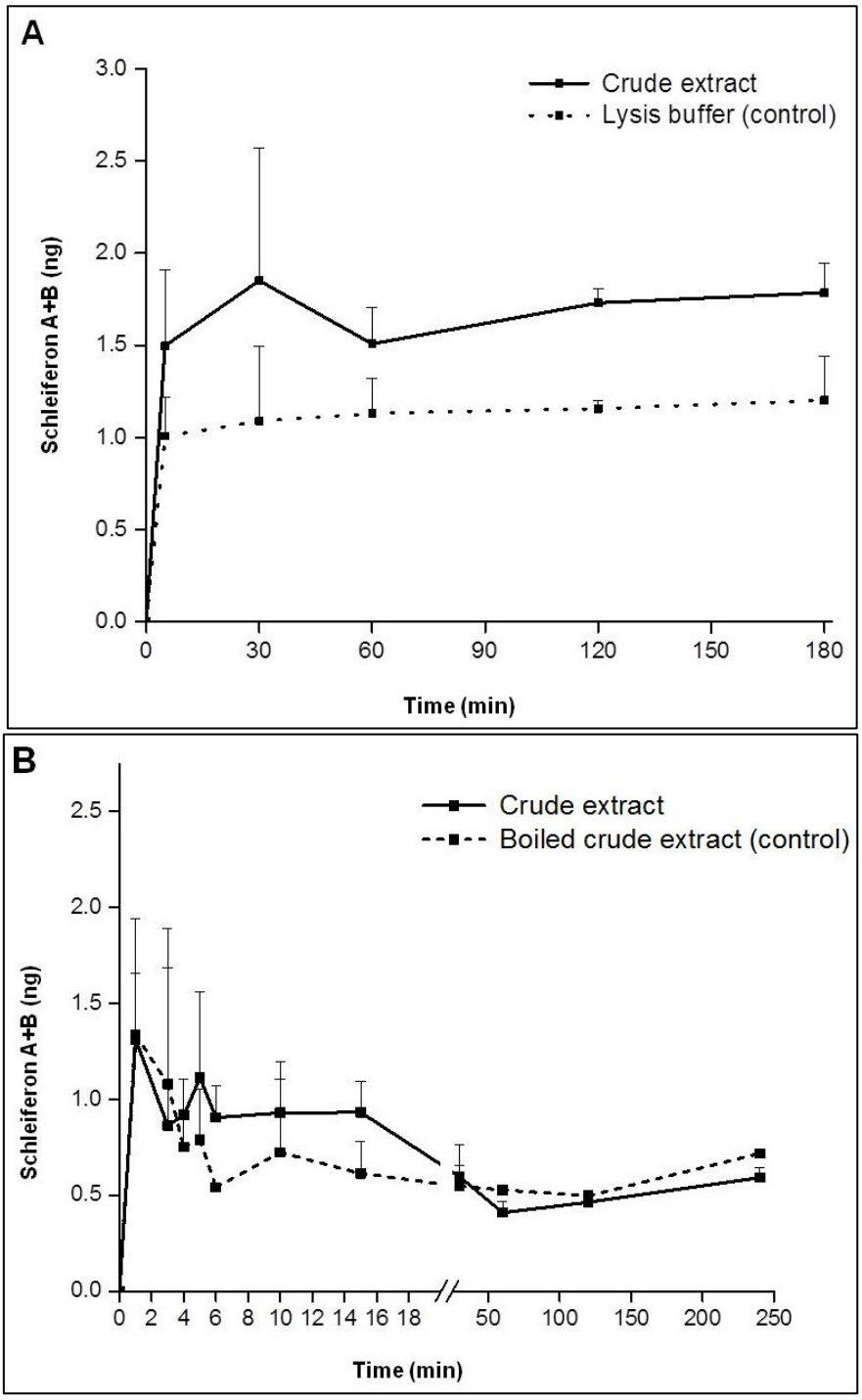

Figure 9: In vitro biosynthesis of schleiferon A and B.

The substrates (phenylethylamine and acetoin) were added to the bacterial crude extract and incubated for 5 to 180 min (**A**) or 1 to 240 min (**B**) at 37 °C. At indicated time points the reaction in each tube was stopped and schleiferon A and B were measured by GC/MS. The quantification was done by co-injection of the internal standard (nonyl acetate). In the control reaction, the crude extract was replaced by lysis buffer (**A**) or was boiled for 5 min at 95 °C (**B**). Data are the means of 3-5 independent experiments and bars indicate mean standard deviation.

1.6. Summary

It is now well known that microorganisms are able to interact with the environment and with organisms of their habitat by producing volatile compounds. They are a very important group of compounds in mediating interactions because they are able to act not only at the site of the production but, also at long distance due to their low molecular weight, high vapour pressure and low boiling point. Although the number of studies regarding microbial volatiles and the interest of researchers in the field increase considerably over the years, all the information available on microbial volatile compounds and their producers were still scattered in the literature. Therefore, we developed mVOC database, which is a user-friendly compilation of existing data on volatile emission from bacteria and fungi, extracted from the literature and available online at http://bioinformatics.charite.de/mvoc/. It is the first online database containing information about mVOCs and their emitting organisms. It is then an indispensable platform to promote and facilitate crosstalk between scientists working in this fast-growing research field. It will also facilitate the study of microbial volatiles and help to better understand the biological and ecological roles as well as the applications of microbial volatiles. By data mining, mVOC database highlights for example the diversity and specificity of microbial volatile compounds. It was shown that bacteria might be capable of synthesizing structurally more diverse volatiles than fungi. Alcohols, ketones and carboxylic acids are the most frequently emitted chemical classes with 2-phenylethanol, 3-methylbutan-1-ol, dimethyl disulfide and dimethyl trisulfide being significantly more often released by bacteria and 1-octen-3-ol the most frequently emitted fungal volatile. It was also shown that the most listed species in the database were isolated from plants, aquatic environments and soil. However, meta-analysis approaches revealed that habitat specificity has little or almost no influence on VOC spectra of microorganisms, confirming the fact that it is not the location of isolation, but rather the nutritional supply and metabolic capabilities of the microorganism that are relevant for it volatile profiles. Data mining also revealed that microorganisms belonging to the rhizosphere might preferentially produce volatile signals, including many of the volatiles known to be emitted by plant roots and that these volatile signals might then specifically modulate the growth and fitness of neighbouring plants. However, among the considerable number of compounds produced by bacteria and fungi described in mVOC database (1200), the biological and/or ecological roles of only ca. 7 % of single volatile has been described so far. They mainly influence insect behaviours as well as plant or microbial growth.

The described functions clearly indicated that one volatile can be produced by numerous microorganisms to a different end while some mVOCs can be produced for the same functions. However, in an ecological relevant situation, mVOCs are produced in a complex volatile-base interaction networks and understanding their functions and specificity need to consider the context in which they are produced.

The diverse functions and properties of mVOCs can be exploited in various biotechnological applications in several areas of industrial, agricultural or medical fields. The studies of microbial volatiles also represent an essential route in bioprospecting, with the promise of discovering novel structures of natural products for novel applications.

Indeed, when analysing volatiles of skin bacteria, two new natural compounds were found and they are promising candidates for further investigations to determine their relevance in antibiotic and anti-virulence therapies. Since the skin is the part of the body, which serves as barrier and protect us again microbial invasion from the external environment, we were interested on the role of mVOCs in skin microbial interactions and defences. We analysed the volatile profile of bacteria belonging to the dominant families of microorganisms naturally found on the skin (*Corynebacteriaceae* and *Staphylococcaceae*). The two families of bacteria were completely separated according to their volatile profile and this could be explained by their metabolism which is different although they were analysed under the same conditions. Among the Staphylococci, *S. schleiferi* isolates showed an interesting volatile profile with more than 30 compounds, which were mainly ketones. Among this volatile bouquet, new natural compounds named schleiferons A and B (3-(phenylamino)butan-2-one and (*E*)-3-(phenylimino)butan-2-one, respectively) were identified and structurally elucidated. Schleiferons A and B inhibited in a concentration dependant manner the growth of Gram-positive bacteria as well as the quorum-depending phenotypes of Gram-negative bacteria. The inhibition of the phenotypes in Gram-negative bacteria was due to reduction of the biosynthetic gene expression and regulation may act at the downstream end of the quorum sensing phosphorylation cascade. These results highlight the role of mVOCs in skin bacteria-bacteria interactions, suggesting their implication in skin microbiota modulation, which might ultimately, affects health and diseases conditions.

2. Results

The presented original data have been published in four publications in English.

2.1. Lemfack, M.C., Nickel, J., Dunkel, M., Preissner, R., Piechulla, B. (2014) mVOC: a database of microbial volatiles. Nucleic Acids Res. 42, 744-748.

2.2. Schenkel, D., **Lemfack, M.C.**, Piechulla, B., Splivallo, R. (2015) A meta-analysis approach for assessing the diversity and specificity of belowground root and microbial volatiles. Front. Plant Sci. 6, 707.

2.3. Piechulla, B., **Lemfack, M.C.** (2016) Microbial volatiles and their biotechnological applications. In: Arimura, G., Maffei, M (eds), Plant Specialized Metabolism: Genomics, biochemistry and biological function. CRC Press 10, 239 - 256.

2.4. Lemfack, M.C., Ravella, S.R., Lorenz, N., Kai, M., Jung, K., Schulz, S., Piechulla, B. (2016) Novel volatiles of the skin-borne bacteria inhibit the growth of Gram-positive bacteria and affect quorum-sensing controlled phenotypes of Gram-negative bacteria. Syst. Appl. Microbiol. 39, 503-515.

2.1. Lemfack, M.C., Nickel, J., Dunkel, M., Preissner, R., Piechulla, B. (2014) mVOC: a database of microbial volatiles. Nucleic Acids Res. 42, 744-748.

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mVOC: a database of microbial volatiles

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ABSTRACT

Scents are well known to be emitted from flowers and animals. In nature, these volatiles are responsible for inter- and intra-organismic communication, e.g. attraction and defence. Consequently, they influence and improve the establishment of organisms and populations in ecological niches by acting as single compounds or in mixtures. Despite the known wealth of volatile organic compounds (VOCs) from species of the plant and animal kingdom, in the past, less attention has been focused on volatiles of microorganisms. Although fast and affordable sequencing methods facilitate the detection of microbial diseases, however, the analysis of signature or fingerprint volatiles will be faster and easier. Microbial VOCs (mVOCs) are presently used as marker to detect human diseases, food spoilage or moulds in houses. Furthermore, mVOCs exhibited antagonistic potential against pathogens *in vitro*, but their biological roles in the ecosystems remain to be investigated. Information on volatile emission from bacteria and fungi is presently scattered in the literature, and no public and up-to-date collection on mVOCs is available. To address this need, we have developed mVOC, a database available online at http://bioinformatics.charite.de/mvoc.

INTRODUCTION

Microorganisms are universal in the biosphere. They are often found in large quantities and diverse compositions (microbiome). For example, there are more microorganisms (~2 kg) than human cells in humans, and most of them are essential and useful for the human host vitality (1). Bacteria are also dominant inhabitants of the leaf surfaces (10^7 cells/cm^2) and they are prominent in the soil, e.g. 1 g of soil contains ~10^{11} microbial cells (2).

It is well known that microbes produce a diversity of natural compounds, e.g. antibiotics. Interestingly, the small molecular mass substances released by microorganisms were often overlooked, partially due to the lack of appropriate absorption and detection technologies. Many of these small molecules (<300 Da) exhibit high-vapour pressures and low boiling points, and, together with a lipophilic character, these features support volatility.

In the past decade, research on microbial smells experienced a renaissance owing to their global appearance. Some examples of bacterial volatile emissions are mentioned here. Undoubtedly, prominent malodorous volatiles are produced by microorganisms during the process of putrefaction (e.g. amines, sulphur compounds, indole and ammonia) (3), whereas the aromas of wines, sauerkraut, cheese and other milk product fermentations are usually recognized as pleasant by human noses (e.g. acids, alcohols and esters). The earthy and muddy smell of wet forest soils is due to the emission of the volatile geosmin released by *Streptomyces* species (4–6). Microbiologists typically recognize the characteristic smell of indole from *Escherichia coli*. The human microbial flora at any given anatomical site is relatively specifically accompanied by a typical volatile organic compound (VOC) profile (e.g. oral and breath malodour, smell of sputum VOCs, gases released by the gut, sweat and sebum smell and foot odour). The VOC mixture of breath originates from more than one source within the respiratory system (e.g. tongue, oropharynx and bronchioles), and respiratory disorders can result in odorous gases being expelled into the air, which can be useful for diagnostic purposes (3). For example, to detect *Mycobacterium tuberculosis*, methyl nicotinate showed promising results to be used as a non-invasive and rapid diagnostic tool, or the emission of 2-nonanone of Pseudomonas aeruginosa VOCs may be used as *in vivo* marker to detect lung infections (7). Freshly secreted sweat is sterile, but due to biotransformation by

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.

microorganisms (aerobic coryneforms, propionibacteria and Micrococcaceae), odoriferous VOCs are produced. Another example is wound infection: when a wound is formed, it is a new ecological niche favourable to microbial growth. Research is ongoing to develop a 'wound sniffing' device that discriminates between wound-related and -unrelated volatiles. Volatiles, particularly off-flavours, are also fingerprints to screen systematically for spoiled foodstuff or to identify hidden microbial growth in buildings (8,9). Furthermore, in the past few years, the interest of researchers studying the effects of microbial VOCs (mVOCs) on plants has become increasingly evident, showing that some rhizobacteria release a blend of volatile components that promote growth of *Arabidopsis thaliana* (10), whereas others inhibit or are toxic for plants (11–14). It is also well documented that mVOCs are important factors in mediating specific microbial interactions; in fact, intra- or inter-species interactions between bacteria and fungi in the soil result in morphological and phenotypical alterations of the receiving organism (15). There are numerous instances of mVOCs being closely associated with insect feeding behaviours, and other microbial volatiles are also known as powerful repellants (16). Moreover, some volatile compounds such as higher alcohols (2-methyl-1-butanol, 3-methyl-1-butanol and isobutanol) can be used as biofuels (17); however, considering the fact that the natural microbial production rates are too low to support industrial production, metabolic engineering is widely used to improve the production (17,18, http://dx.doi.org/10.5772/52050).

These are just a few examples of the up to now known 349 bacteria and 69 fungi that are volatile emitters. Taking in considerations that to date ~10 000 microbial species are described and at least a million are expected to exist on earth, the VOC profiles of a surprisingly small number of microorganisms were investigated so far. The VOC spectra of microorganisms are species-specific and can be simple or complex (19). The qualitative and quantitative composition of an mVOC profile is variable, depending on growth conditions (temperature, oxygen availability, pH), the carbon source availability and the age of the culture (3,20–24). Ultimately, the volatile emission profile is a consequence of specific metabolic activities of the particular microorganism.

Considering the importance and the central roles of mVOCs in our biosphere, our objective was the establishment of a database of microbial volatiles for public use. Here, we present for the first time a user-friendly compilation of the microbial volatiles extracted from the literature. Originally, only mVOCs were filed using the Pubchem ID as an essential criterion. During the literature search, it turned out that many mVOCs have not received a Pubchem ID, but might be biologically relevant. Therefore, these compounds were also included into the database. Mixtures of mVOCs are composed of various chemical classes, e.g. low molecular weight fatty acids and their derivatives (hydrocarbons, alcohols, aldehydes and ketones), terpenoids, aromatic compounds, nitrogen containing compounds and volatile sulphur compounds (15,25). To date, ~1000 volatiles are filed in the mVOC database. References are given to each bacterial and fungal strain or isolate presented in the database. The user interface offers several search options, for instance, by species name, Pubchem ID, structure, molecular weight and logP value. Online upload is possible, allowing a timely incorporation of new data sets, which is expected to happen progressively in this fast-growing research field.

MATERIALS AND METHODS

The data were acquired by an extensive literature search available on PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Most of the information on mVOCs was found in ~20 journals and the full-text of ~100 articles was yielding most of the results compiled in the mVOC database. The literature was manually screened by biochemists. To update mVOC in the future, literature will continuously be screened and data will be checked by a chemist or biochemist before entering them manually to the database.

For conducting the similarity search of the compounds, the chemoinformatics package MyChem (http://mychem.sourceforge.net/) is integrated into the database. It enables the analysis and conversion of chemical data using OpenBabel (http://openbabel.org/) functionality. For the purpose of calculating the Tanimoto coefficient (26), it is obligatory to assign fingerprints to the compounds. This step is also performed by the MyChem package. Thereto, OpenBabel uses the Daylight theory for fingerprints (http://www.daylight.com/dayhtml/doc/theory/theory.finger.html). For the similarity determination between the compound of interest and the compounds of the mVOC database, the Tanimoto coefficient is the measure of choice:

$$\text{Tanimoto coefficient}_{A,B} = \frac{AB}{A+B-AB}$$

Bits of the binary fingerprint vectors were set to one in compound A and compound B as well as bits were set to one in both compounds and used for the calculation. The values calculated by the Tanimoto coefficient range between 0 and 1, where 1 indicates similar structures and 0 means that no similarity is found between the fingerprint representations of the molecules. Referring to the 'similarity property principle' (27), compounds that are structurally similar should exhibit a similar biological function. Nevertheless, small structural modifications can change the biological activity of the molecules dramatically (28). However, a Tanimoto coefficient >0.85 implies that the compared compounds may have a similar biological activity (29).

As an applet for sketching compounds for the 'Structure Search' and 'Add a new mVOC' function, the open-source web-component ChemDoodle is implemented on the mVOC website. ChemDoodle is also used for a 3D visualization of the mVOC structure. ChemDoodle guarantees smooth usage on different platforms.

For retrieving Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, a similarity search between the 846 mVOCs and the 200 000 compounds from the SuperTarget database (30) was carried out. This step was conducted to obtain information on synthesis/degradation

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of the mVOCs as well as potential target interactions. Compounds were considered when the Tanimoto coefficient of the similarity pair between volatile and SuperTarget compound was at least 0.85. Those compounds were mapped onto the pathway maps. Additionally, mVOCs were mapped to KEGG compounds and are also displayed on the pathway maps. The database features pathways of species available in the database. The mapped pathways are visualized by web service.

The mVOC database is implemented as a relational database on a MySQL server. Php and javascript have been used to build the website. Web access is enabled by Apache HTTP Server 2.

RESULTS

With a number of 846 compounds and 5431 synonyms, which are assigned to 349 bacterial and 69 fungi species, mVOC is the first online database containing information about mVOCs and their emitting organisms.

Search options

The database provides several possibilities to search for compounds (Figure 1). On the one hand, a form ('browse mVOC') is available, and the user can choose to search by PubChem-ID, name or molecular formula. In addition, compounds can be searched by selecting a

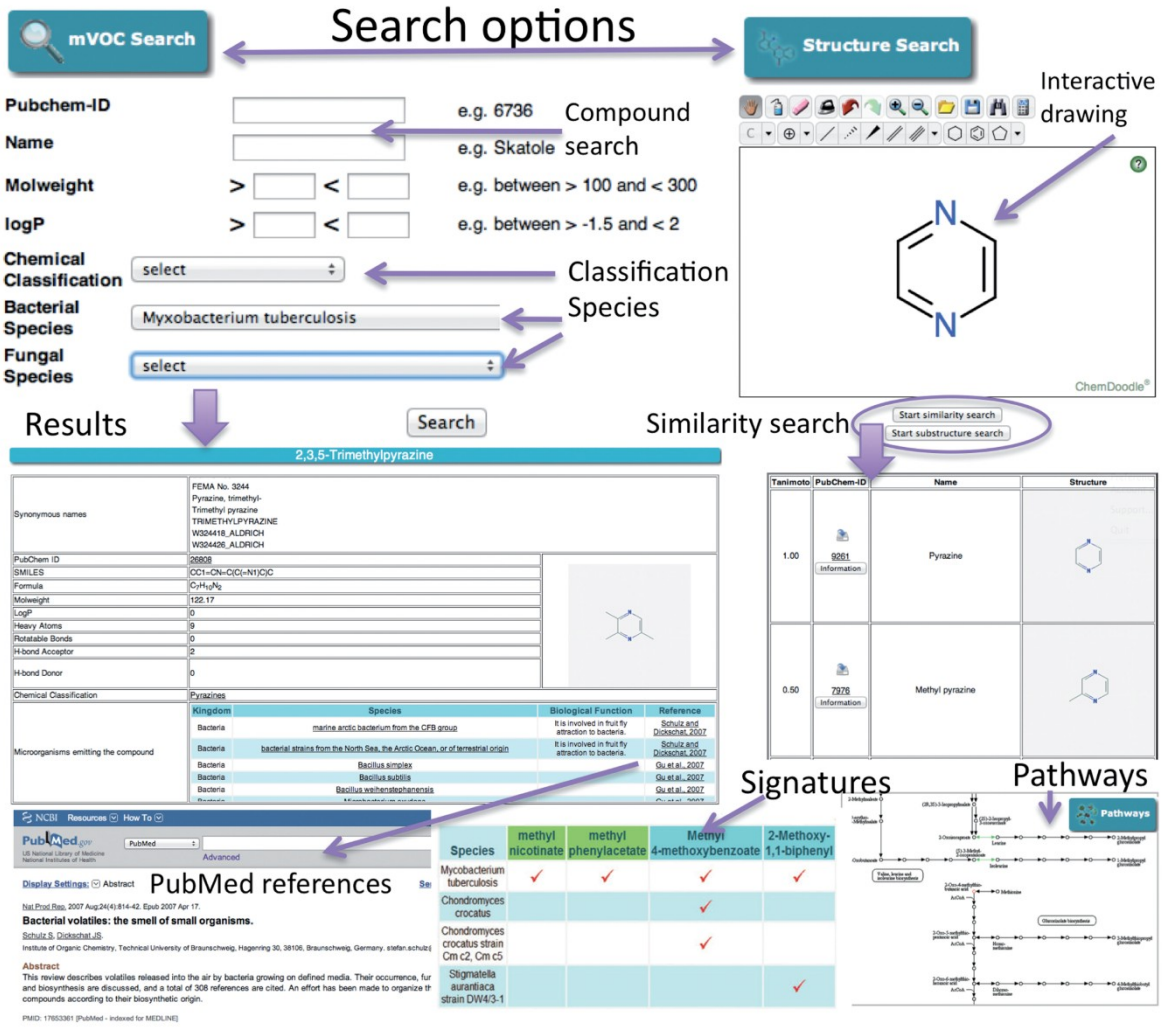

Figure 1. The mVOC database offers different search options. mVOC search: a general search form for mVOCs based on PubChem ID, name, several molecular properties as well as species. The result table is directly retrieved. Structure search: interactively drawing a structure and performing a structure or substructure search. The result table shows volatile compounds similar to the search entry (similarity search) or volatile compounds including a substructure that is similar to the search entry (substructure search). By clicking on 'Information', one will be directed to the result table of the mVOC. Signatures: the signature table shows all species emitting the same compounds as the chosen species. Compounds emitted by just one species are highlighted in green. KEGG pathways: cutout of the 2-oxocarboxylic acid metabolism pathway.

range of properties like molecular weight, logP values or by specific chemical groups (e.g. alkenes), by species, by the microorganisms' kingdom and by a combination of these parameters. Furthermore, compounds can be searched by structural similarity ('Structure Search') including substructure search. For this purpose, a molecular structure is necessary to be known. The compound of interest is screened against the database by calculating the Tanimoto coefficient between the composed compound and all compounds of the mVOC database. Finally, the user can draw a compound with the embedded ChemDoodle interface (http://www.chemdoodle.com) or upload its MOL file. In addition to that, a possibility of browsing the mVOC database is given under the form 'browse mVOC'. The database can be browsed by initial letters or chemical groups.

Search results

The resulting report of 'browse mVOC' shows information about the compounds including name, synonyms, Pubchem-ID and structural information of the mVOC of interest (Figure 1). Additionally, microorganisms emitting the compound, the effect of the compound on other organisms, the respective methods for retrieving the compounds and the corresponding references are displayed. The search results for the 'Structure Search' are represented in order of similarity with information given about the calculated Tanimoto coefficient, PubChem-ID, name and 2D structure. The button 'Information' provides detailed information about the mVOCs that are similar to the query compound.

Biological interpretation

The website features the use of KEGG pathway maps (http://www.kegg.jp/) through Web service. KEGG pathway maps supply knowledge about metabolic pathways as well as compound target interactions and offer a possibility for biological interpretation (Figure 1). Compounds of the mVOC database are mapped onto the pathways showing information about metabolic pathways providing an opportunity for further analysis. Moreover, an investigation of medical effects is also possible. A link to gene or gene clusters responsible for mVOC production will be included in future versions of this database.

Another important feature is a 'signature table' of an organism of choice (Figure 1). After selecting a species from the bacterial or fungal species dropdown menu from 'browse mVOC', a 'signature' button is available on top of the result page. The 'signature table' plots the emitted mVOCs of the chosen species compared with all microbial species, which emit these mVOCs. The table shows the uniqueness of the compounds, which is, for example, important for distinguishing between (more or less pathogenic) species.

Database extension

To enlarge the mVOC database, an upload function 'Add new mVOCs' is included (Figure 1). The user can upload a compound by drawing its structure with the ChemDoodle application. After uploading the compound, it will be verified by biochemists, and after being proofed as mVOC, it will be included into the database. Users are also encouraged to contact the authors when new volatile spectra are ready to be uploaded.

DISCUSSION

Microbes make up the majority of the world's biomass; their numbers and diversity greatly surpass those of all other organisms (31). Microbial chemical ecology is an important part of our life, and the analysis of the microbiome and unravelling its physiology including immune response, metabolism as well as pathology are future goals (32). Although sequencing becomes cheaper, an analysis of volatiles will always be faster and less invasive. Therefore, the mVOC database is an indispensable platform for this burgeoning field of microbial volatiles. Interest is particularly focussed on the identification of 'signature volatiles' of human, animal and plant pathogenic species (33). Based on these results, new possibilities for using diagnostic tools can be considered. The application of volatile antibiotics can also be envisioned.

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A meta-analysis approach for assessing the diversity and specificity of belowground root and microbial volatiles

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Volatile organic compounds are secondary metabolites emitted by all organisms, especially by plants and microbes. Their role as aboveground signals has been established for decades. Recent evidence suggests that they might have a non-negligible role belowground and might be involved in root–root and root–microbial/pest interactions. Our aim here was to make a comprehensive review of belowground volatile diversity using a meta-analysis approach. At first we synthesized current literature knowledge on plant root volatiles and classified them in terms of chemical diversity. In a second step, relying on the mVOC database of microbial volatiles, we classified volatiles based on their emitters (bacteria vs. fungi) and their specific ecological niche (i.e., rhizosphere, soil). Our results highlight similarities and differences among root and microbial volatiles and also suggest that some might be niche specific. We further explored the possibility that volatiles might be involved in intra- and inter-specific root–root communication and discuss the ecological implications of such scenario. Overall this work synthesizes current knowledge on the belowground volatilome and the potential signaling role of its constituents. It also highlights that the total diversity of belowground volatiles might be orders of magnitude larger that the few hundreds of compounds described to date.

Keywords: microbes, fungi, bacteria, volatiles, diversity, rhizosphere, mycorrhizas, roots

Introduction

Secondary metabolites are small molecules that are produced by all living organisms. Unlike primary metabolites which are directly involved in regular growth and development, secondary metabolites might be produced only at specific developmental stages or under certain circumstances; hence they might provide a functional readout of cellular state (Patti et al., 2012). Tens of 1000s of secondary metabolites derived from plants and microbes are known to humans as drugs, food additives or flavors, and fragrances; yet, their ecological functions remain poorly understood.

Secondary metabolites indeed play a central role in inter-organismic interactions. In numerous cases volatile and non-volatile secondary metabolites have been implicated in defense

and communication among organisms. Recently, volatiles have attracted sustained attention, especially in belowground communication, due to their ability to travel further distances than non-volatile metabolites (Rasmann et al., 2005; Wenke et al., 2010; Peñuelas et al., 2014). Because of their potent biological activities on plants, the use of volatiles in agriculture have been suggested as a possible alternative to pesticides (Bitas et al., 2013; Kanchiswamy et al., 2015). A search through literature and databases allows estimating the known structural diversity of volatiles derived from plant flowers – about 1700 volatiles from 991 species (Knudsen et al., 2006; Dunkel et al., 2009) – and from microbes, including fungi and bacteria – 1093 volatiles from 491 microbes at the time of this study (Lemfack et al., 2014). Yet considering that 10^7–10^9 bacterial species (Schloss and Handelsman, 2004), 1.5 million fungal species (Hawksworth, 2001) and 2,98,000 of plant species (Mora et al., 2011) might exist on earth, the number of volatiles will increase as new species are being characterized and discovered.

In the past 5 years, the ecological role of volatiles in above- and belowground interactions among plants, fungi, bacteria, and insects has been addressed in a series of comprehensive reviews (Wenke et al., 2010, 2012; Bailly and Weisskopf, 2012; Effmert et al., 2012; Davis et al., 2013; Farag et al., 2013; Audrain et al., 2015; Kanchiswamy et al., 2015; Schmidt et al., 2015). The latest of these reviews (Kanchiswamy et al., 2015) covered literature up to the beginning of 2015. Most recently a further example of belowground volatile based communication has been brought to light for plants and the ectomycorrhizal fungus *Laccaria bicolor* (Ditengou et al., 2015). Some volatile sesquiterpenoids emitted by the latter fungus were shown to induce root branching in poplar, a host plant which can enter into symbiotic interactions with the fungus, but also in *Arabidopsis*, a non-host plant unable of symbiosis with *Laccaria*. Remarkably not all fungal sesquiterpenoids induced root branching: the volatile (–)-thujopsene was implicated in the root morphological change but β-caryophyllene, another sesquiterpenoid also emitted by maize roots (Rasmann et al., 2005), had no effect on branching. These observations raise questions about the specificity of belowground signals as well as the ability of the target organisms to perceive and react to volatiles.

Soil is actually a highly colonized inhomogeneous substrate. Non-homogeneity is not only reflected in terms of structure and porosity but also in terms of nutritional differences (Schoenholtz et al., 2000). Besides, organisms present in the soil might also provide specific niches for defined microbes, thus exerting a community structuring effect. Belowground community structuring has indeed been observed in numerous cases. A textbook example includes root nodules in legumes which are exclusively colonized by nitrogen fixing rhizobacteria (Gage, 2004). More recent examples are provided by *Arabidopsis*' root endophytic microbial community made of *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (Bulgarelli et al., 2012) and by truffle's fruiting bodies which host bacterial communities clearly distinct from those of the surrounding soil (Antony-Babu et al., 2014). This belowground community structuring might explain why some volatiles could act as successful signaling cues within such communities, however evidence that specific volatiles are emitted in defined habitats/niches is currently limited.

The aim of this paper is to quantify the diversity and explore the specificity of belowground volatiles produced by microbes and plant roots. For this purpose we synthesized existing literature on plant root volatiles and relied on the "mVOC database" of microbial volatiles (Lemfack et al., 2014) to address questions such as: how structurally diverse are plant root and microbial volatiles? Which volatiles are common and specific to microbes and plant roots? Is their emission influenced by microbial phylogeny or habitat; and finally do root volatiles serve as signals for neighboring plants? Overall our aim was to shed more light on belowground volatiles diversity and functions by essentially using a quantitative approach to diversity and by integrating information on the phylogeny and the habitat of the emitters.

Materials and Methods

Diversity of Plant Root Volatiles

Volatile organic compounds (VOCs) released by plant roots have been investigated in a limited number of species. Here we gathered information relative to volatile diversity in barely – *Hordeum vulgare* – (29 compounds; Gfeller et al., 2013), the model plant *Arabidopsis thaliana* (eight compounds; Steeghs et al., 2004), maize – *Zea mays* (one compound; Rasmann et al., 2005) and the bean *Vicia faba* (one compound; Babikova et al., 2013). Overall these plant roots emitted 39 volatiles, which have been grouped in **Figure 1** based on their biosynthetic origins/chemical classes (i.e., terpenoids, alcohols).

Diversity of Microbial Volatiles

The diversity of microbial volatiles was investigated using the mVOC database (Lemfack et al., 2014). At the time of this study, the database comprised 1093 volatiles emitted by 135 fungi and 356 bacteria. As for plant roots, volatiles were classified according to chemical classes/biosynthetic origins (**Figure 2**).

Specificity of Microbial Volatiles Linked to Taxonomy and Ecological Niches

To understand how specific or common volatiles were in microbes, bacteria and fungi of the mVOC database were classified in taxonomical units either at the phylum or class level. Gaining insight into niche specificity was achieved by classifying the microbes of the mVOC database based on their habitat. Because of our focus on belowground interactions, classification was made in five categories: fungi or bacteria living in the rhizosphere, fungi, or bacteria living in the soil (excluding the rhizosphere), and microbes living in any other habitat (i.e. animals, marine habitats, and microorganisms associated to above-ground plant parts). Classification in specific niches/habitats was based on various data sources which will be shortly included in the mVOC database. Because we were interested in habitat/niche specificity, microbes which were ubiquitous to more than one habitat/niche were excluded from the analysis.

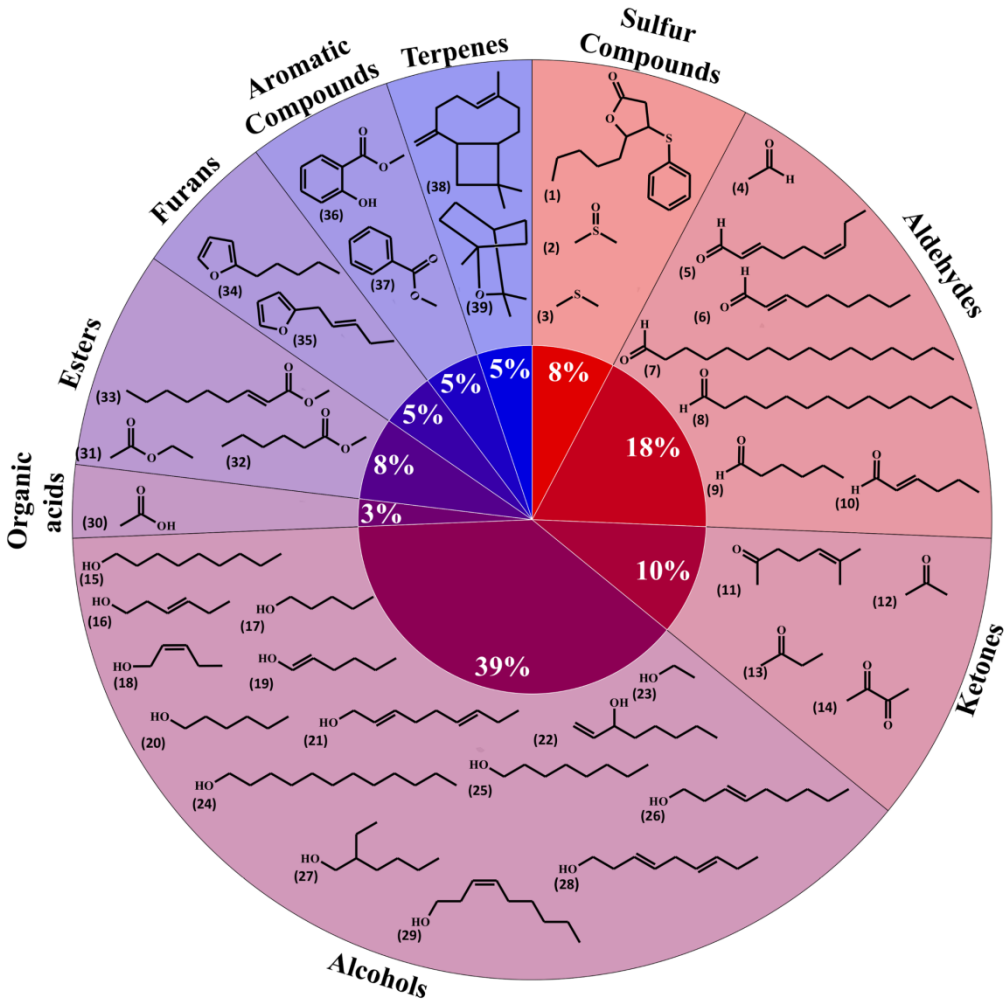

FIGURE 1 | Diversity of plant root volatiles. A total of 39 volatiles released from barley, maize, *Arabidopsis* or fava bean roots were classified based on their chemical classes and shown here in a pie chart. 5-Pentyl-4-phenylsulfanyloxolan-2-one (1); dimethyl sulfoxide (2); dimethyl sulfide (3); acetaldehyde (4); (2*E*,6*Z*)-nona-2,6-dienal (5); (*E*)-non-2-enal (6); hexadecanal (7); tetradecanal (8); hexanal (9); (2*E*)-hex-2-enal (10); 6-methylhept-5-en-2-one (11); acetone (12); butan-2-one (13); butane-2,3-dione (14); nonan-1-ol (15); (3*E*)-hex-3-en-1-ol (16); pentan-1-ol (17); (2*Z*)-pent-2-en-1-ol (18); (1*E*)-hex-1-en-1-ol (19); hexan-1-ol (20); nona-2,6-dien-1-ol (21); oct-1-en-3-ol (22); ethanol (23); dodecan-1-ol (24); octan-1-ol (25); (*E*)-non-3-en-1-ol (26); 2-ethylhexan-1-ol (27); nona-3,6-dien-1-ol (28); (*Z*)-non-3-en-1-ol (29); acetic acid (30); ethyl acetate (31); methyl hexanoate (32); methyl (*E*)-non-2-enoate (33); 2-pentylfuran (34); 2-pent-2-enylfuran (35); methyl salicylate (36); methyl-benzoate (37); β-caryophyllene (38); 1,8-cineole (39).

Effect of Neighboring Plant on Root Development

The influence of neighboring plants on root development was investigated by compiling data from 18 publications (Mahall and Callaway, 1992; Gersani et al., 2001; Maina et al., 2002; Day et al., 2003; Falik et al., 2003, 2006; Gruntman and Novoplansky, 2004; O'Brien et al., 2005; Dudley and File, 2007; Murphy and Dudley, 2007, 2009; Semchenko et al., 2007, 2014; Broz et al., 2008; Milla et al., 2009; Fang et al., 2013; Schmid et al., 2013a). In all those works root development (biomass or root length depending on the parameter reported) of a plant subjected to neighboring plants was compared to root development of a plant without neighbors. The effects on roots were classified as "increase, decrease, no effect" based on the statistics reported in the papers. Subject plants were grouped either based on genetic relatedness with the interacting plants (as kin, conspecific but not kin, and foreign species) or as monocots and dicots. Cases in which the kinship of individuals of the same species was unspecified were categorized as conspecific.

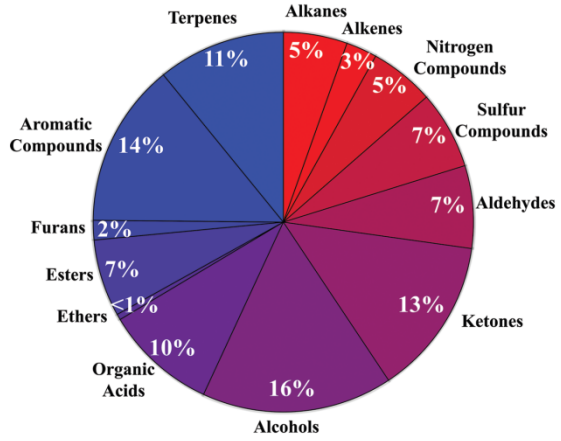

FIGURE 2 | Diversity of microbial volatiles. The 1093 volatiles from the mVOC database (Lemfack et al., 2014) grouped in chemical classes are shown as a pie chart.

Results

Diversity of Plant Root and Microbial Volatiles

Whereas publications investigating volatiles emitted by aboveground plant organs abound, only a few papers have been published on root volatiles, most likely due to the technical difficulties in sampling volatiles in soil matrices. Compiling the information from root volatiles emitted by maize (Rasmann et al., 2005), barley (Gfeller et al., 2013), *Arabidopsis thaliana* (Steeghs et al., 2004), and the bean *Vicia faba* (Babikova et al., 2013) revealed an overall diversity of 39 volatiles belonging to nine chemical/biosynthetic groups (**Figure 1**). With 66% of all volatiles, alcohols, aldehydes, and ketones represented the major share of root volatiles. The remaining 44% was composed of minor groups (sulfur compounds, terpenoids, aromatic compounds, furans, esters, and organic acids) each represented by a single or two compounds. By contrast to the scarce information on root volatiles, microbial volatiles have been investigated more thoroughly. An effort to synthesize the large amount of information on microbial volatiles has recently been made through the mVOC database (Lemfack et al., 2014), which also served as the basis of the present study. Here a total of 1093 microbial volatiles from the mVOC database have been grouped according to chemical classes/biosynthetic pathways and the resulting data is presented as a pie chart in **Figure 2**. Even though some volatiles like ketones, esters, sulfur-containing compounds, and furans appeared with a comparable frequency as in plants roots and microorganisms, the microbial volatilome comprised a greater structural complexity of organic acids, aromatic compounds, and terpenes than plant roots, at least considering the currently available data (**Figures 1** and **2**). Five groups of microbial volatiles (terpenes, alcohols, ketones, aromatic compounds, and organic acids) represented each 10% or more of the volatiles, overall accounting for 64% of the total diversity. Aldehydes, sulfur and nitrogen containing compounds, alkanes, alkenes, furans, ester, and ethers represent minor groups accounting together for almost 37% of the total diversity.

Which Microbes Produce Plant Root Volatiles?

A total of 28 plant root volatiles were also produced by microbes. These volatiles included 11 alcohols (dodecan-1-ol; ethanol; 2-ethyl-1-hexanol; hexan-1-ol; 2-hexen-1-ol; 3-hexen-1-ol; 1-nonanol; 1-octanol; 1-octen-3-ol; pentanol; 2-penten-1-ol), 4 aldehydes (acetaldehyde; hexanal; 2-hexenal; tetradecanal), two aromatic compounds (methyl benzoate; methyl salicylate), two esters (ethyl acetate; methyl hexanoate), one furan (2-pentylfuran), four ketones (acetone; butanone; butanedione; 6-methyl-5-hepten-2-one), one organic acid (acetic acid), two sulfur compounds (dimethyl sulfide; sulfinylbismethane), and one terpene (β-caryophyllene). Our aim was to understand if these volatiles were preferentially produced by specific bacterial or fungal phyla/classes. For this purpose, microbes emitting plant root volatiles were grouped in phyla and in some cases in classes. The heatmap in **Figure 3** represents the percentage of microbes, which are emitters of the plant root volatiles of **Figure 1**.

In terms of volatile groups, 14 fungal and 22 bacterial phyla emitted plant root volatiles at a rather low frequency (<10% as shown from the color scale on the heatmap of **Figure 3**). Alcohols were the most frequent and were emitted by four of the seven bacterial phyla and by all the fungal phyla. Volatiles belonging to remaining groups were similarly emitted at a low frequency by 50% of all phyla (fungal and bacterial). Interestingly all volatile groups occurred in at least one fungal and one bacterial phylum. Furans were produced by a fair percentage of fungi belonging to the three fungal phyla considered here whereas it was emitted at low frequency within a single bacterial phylum (*Firmicutes*, specifically the *Bacilli* class).

Considering the data in terms of phyla highlighted that members of the *Firmicutes* and *Proteobacteria* bacterial phyla and *Ascomycetes* fungi emitted volatiles belonging to most of the chemical groups. Zooming into bacterial classes revealed that among the *Firmicutes*, *Bacilli* were the most frequent emitters of plant root volatiles while among the *Proteobacteria*, β-, and γ-*Proteobacteria* were the most frequent emitters. Interestingly, acetic acid (the only molecule in the category "organic acid") was produced by about 50% of all *Bacilli* and an even higher percentage of *Clostridia* (the highest percentage with Negativicutes reflects the fact that this class has a single representative).

Overall these results highlight that numerous microbes are capable of emitting the same volatiles as plant roots. They also suggest that some phyla might be better than others at producing these volatiles. Bacteria belonging to the *Firmicutes* (*Bacilli*), to the *Proteobacteria* (β- and γ-*Proteobacteria*) and *Ascomycetes* fungi specifically stand out for their ability to produce a large variety of plant root volatiles.

Common and Specific Volatiles to Plant Roots, Bacteria, and Fungi

The microbial volatiles of the mVOC database and the plant root volatiles of **Figure 1** have been presented according to

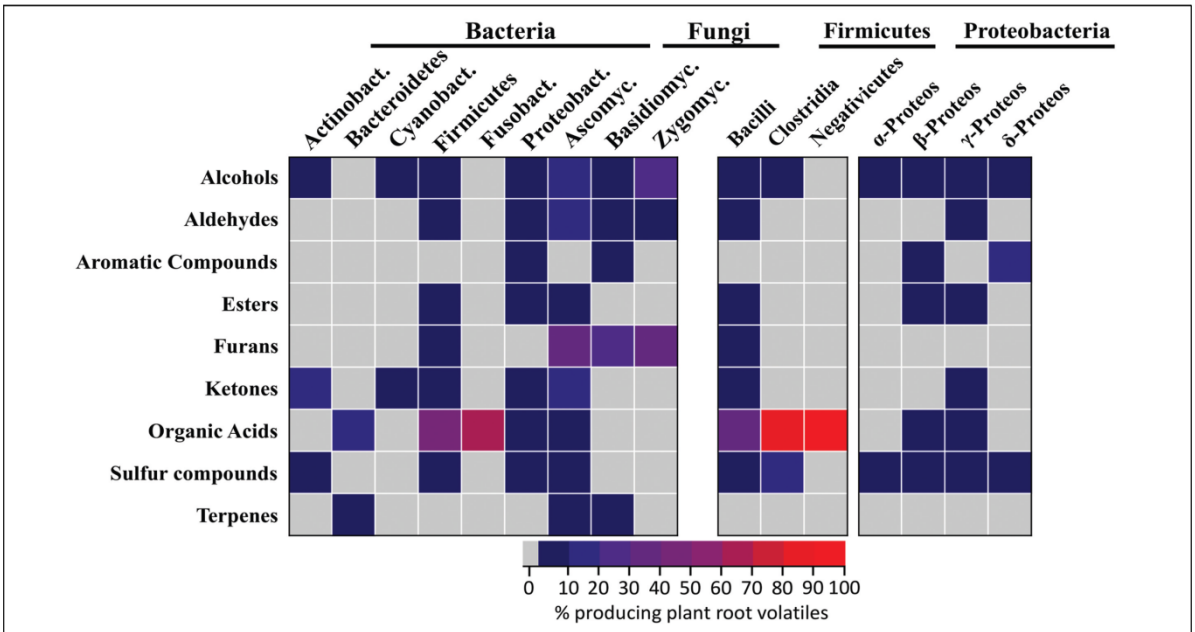

FIGURE 3 | Which microbes produce plant root volatiles? Bacteria and fungi producing the same volatiles as plant roots (**Figure 1**) were identified from the mVOC database. The results are displayed for the different phyla or classes of bacteria and fungi as a heatmap where each cells represents the percentage of microbes emitting volatiles of **Figure 1**. Actinobact: *Actinobacteria* ($n = 64$), *Bacteroidetes* ($n = 44$), Cyanobact: *Cyanobacteria* ($n = 19$), *Bacilli* ($n = 11$), *Clostridia* ($n = 10$), *Negativicutes* ($n = 1$), Fusobact: *Fusobacteria* ($n = 3$), α-Proteos: α-*Proteobacteria* ($n = 25$), β-Proteos: β-*Proteobacteria* ($n = 43$), γ-Proteos: γ-*Proteobacteria* (n = 62), δ-Proteos: δ-*Proteobacteria* ($n = 17$), Ascomyc: *Ascomycota* ($n = 104$), Basidiomyc: *Basidiomycota* ($n = 28$), Zygomyc: *Zygomycota* ($n = 3$).

the potential origin/habitat of their emitters. These origins have been regrouped here in five categories as plant roots (39 volatiles), rhizosphere fungi (261 volatiles), rhizosphere bacteria (209 volatiles), soil fungi (187 volatiles), soil bacteria (483 volatiles). The data is presented as a Venn diagram highlighting the number of specific and common volatiles among groups (**Figure 3**).

A total of 853 volatiles were emitted by plant roots and belowground microbes. Considering the five groups defined here, all groups shared eight volatiles; however, the majority of volatiles were unique to distinct origins/habitats. For example, of the 39 volatiles produced by plant roots (**Figure 1**), 12 (or 31%) were solely produced by roots and not by any other microbes. Depending on their habitat fungi produced 145 (rhizosphere) and 96 (soil) unique volatiles not shared by any other groups; by contrast soil and rhizosphere fungi had 61 volatiles in common. The same argument can be made for bacteria, which produced 76 (rhizosphere) and 297 (soil) unique volatiles, and shared 126 of them.

Overall this data exemplifies the specificity but also the extent of the overlap in volatile signals emitted by plant roots and microbes. It highlights the existence of a core volatilome for bacteria and plant roots but also the fact that a high proportion of volatiles are specific to organisms in defined habitats.

Are Microbial Volatiles Niche Specific?

Fungi and bacteria from the mVOC database were regrouped according to their lifestyle/habitat. Similarly to **Figure 4** three categories were considered in relation to possible interactions with plants: organisms typically found in the soil (S), microbes associated with the rhizosphere (R) and organisms which did not fall in those two categories (N) (i.e., either associated to above plant organs or with animals). Only volatiles occurring in at least 10 microbes are shown here. Values in the heatmap represent the percentage of microbes emitting a specific volatile in each category.

In terms of chemical classes/groups, numerous terpenes, aromatic compounds, nitrogen, and sulfur containing compounds, alkanes and alkenes were predominantly produced by bacteria compared to fungi. Some volatiles such as nitrogen containing compounds were actually almost exclusively produced by bacteria. By contrast no volatiles were exclusively produced by fungi. In most cases habitat specificity (i.e., soil, rhizosphere) seemed to have little influence on volatiles patterns. Volatiles belonging to a few groups were, however, predominantly produced by rhizosphere (R) organisms (in opposite to soil (S) and "other" (N) organisms). This was the case for example in fungi for alcohols, sulfur compounds, some aromatic compounds (i.e., 2-phenylethanol) and some ketones (i.e., octan-3-one). Similarly in bacteria nitrogen containing

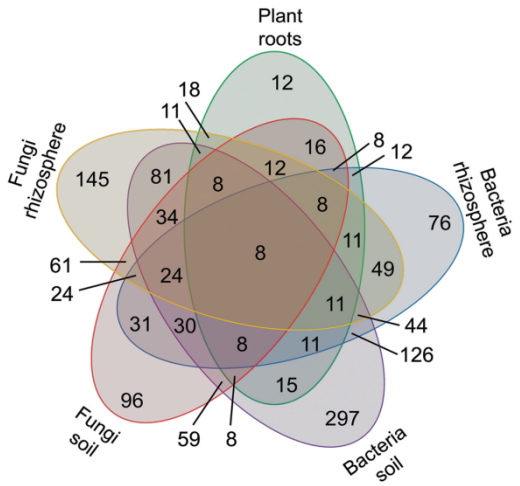

FIGURE 4 | Number of volatiles specific and common to plant roots, bacteria, and fungi. A total of 853 volatiles emitted by plant roots (**Figure 1**) and belowground microbes were grouped based on their source/habitat in the five categories and are displayed in a Venn diagram. The numbers of volatiles specific or shared among categories are displayed here.

compounds production seemed slightly higher in rhizosphere organisms.

Plant root volatiles shown in bold were marked with an asterisk in **Figure 5**. With the exception of 1-octen-3-ol, most of these volatiles were emitted by microbes in most/all categories. Nevertheless it is noteworthy that six of the eight plant root volatiles shown here [2-pentylfuran; dimethyl sulfide (syn. (methylsulfanyl)methane]; ethyl acetate; acetone; ethanol; 1-octen-3-ol) were emitted by a comparatively higher percentage of rhizospheric fungi compared to fungi and bacteria colonizing different habitats.

These results demonstrate marked differences in terms of volatile production patterns among bacteria and fungi. This suggests that bacteria might be capable of synthesizing structurally more diverse volatiles than fungi. They also indicate that microbes belonging to specific niches/habitats, especially to the rhizosphere, might preferentially produce volatile signals, including many of the volatiles also emitted by plant roots.

Could Root Volatiles be Perceived by Neighboring Plants?

There is a mounting body of evidence that neighboring plants can communicate with each other through their roots (Dudley and File, 2007; Bhatt et al., 2011; Fang et al., 2013; Schmid et al., 2013b). Obvious signals for such communication might be volatile molecules. Additionally, volatile emission patterns of aboveground plant organs were shown to be dependent on genetic relatedness. For the sake of clarity, kin plants by definition share the same parents/ancestors, as opposed to conspecific plants which, besides belonging to one species, do not have common parents/ancestors. Recently volatile profiles of kins were shown to be more similar to each other than those of plants without kinship (conspecific plants) (Karban et al., 2014). This led us to question whether plant roots react differently to neighboring plants based on their genetic relatedness (i.e., kins, conspecific but not kins, or foreign (different species) – see cartoon of **Figure 6**). Furthermore we also questioned if differences existed among monocots and dicots.

To answer these questions we gathered publications, which compared root development of one plant with a neighbor to a single plant without neighbor. A total of 30 observations from 18 publications were taken into account and their outcomes have been synthesized in **Figure 6**.

Comparing kins to conspecifics revealed that the roots of more than 50% of kins were unaffected by their neighbors compared to only 21% for conspecifics. When an effect was observed, this predominantly corresponded to an increased root biomass for both categories. The opposite was true for plants subjected to a foreign neighbor. These predominantly (43% of all observations) reacted to the neighbor by decreasing or shortening their roots. Patterns were less obvious with monocots and dicots. Indeed the number of cases in which roots were either affected (increase or decrease in biomass/root length) or unaffected were comparable.

Overall these results highlight that among kin, foreign and conspecific plants, roots of kins are the less likely to be influenced by a neighbor. They also exemplify that plants sharing the same genotype (kins and conspecifics) might predominantly react to each other by increasing their root biomass/root length, while plants with a foreign neighbor might commonly decrease their root biomass.

Discussion

During the past decade VOCs have gained recognition as essential signals in inter-organismic interactions. Especially belowground volatiles might convey information among plant roots, microbes, and insects. The diversity of volatiles and effects on their target organisms have been recently synthesized in a series of comprehensive reviews (Wenke et al., 2010, 2012; Bailly and Weisskopf, 2012; Effmert et al., 2012; Davis et al., 2013; Kanchiswamy et al., 2015). Our aim here was to bring this synthesis one step further by using a quantitative meta-analysis approach and integrating data about phylogeny and potential habitat of the emitters.

Diversity of Belowground Volatiles

Adding up volatiles emitted by plant roots to volatiles emitted by soil/root microbes brings the total diversity of belowground volatiles to 853. Considering the scarce information on plant root volatiles (only a few existing publications), and the huge unexplored diversity of soil microbes, the overall diversity of belowground volatiles might be orders of magnitudes higher than the few 100s of compounds described to date.

A note of caution should nevertheless be used when estimating diversity from literature data, since most studies describing volatiles from microbes or plant roots have been conducted under laboratory (and sometimes axenic) conditions. Indeed it

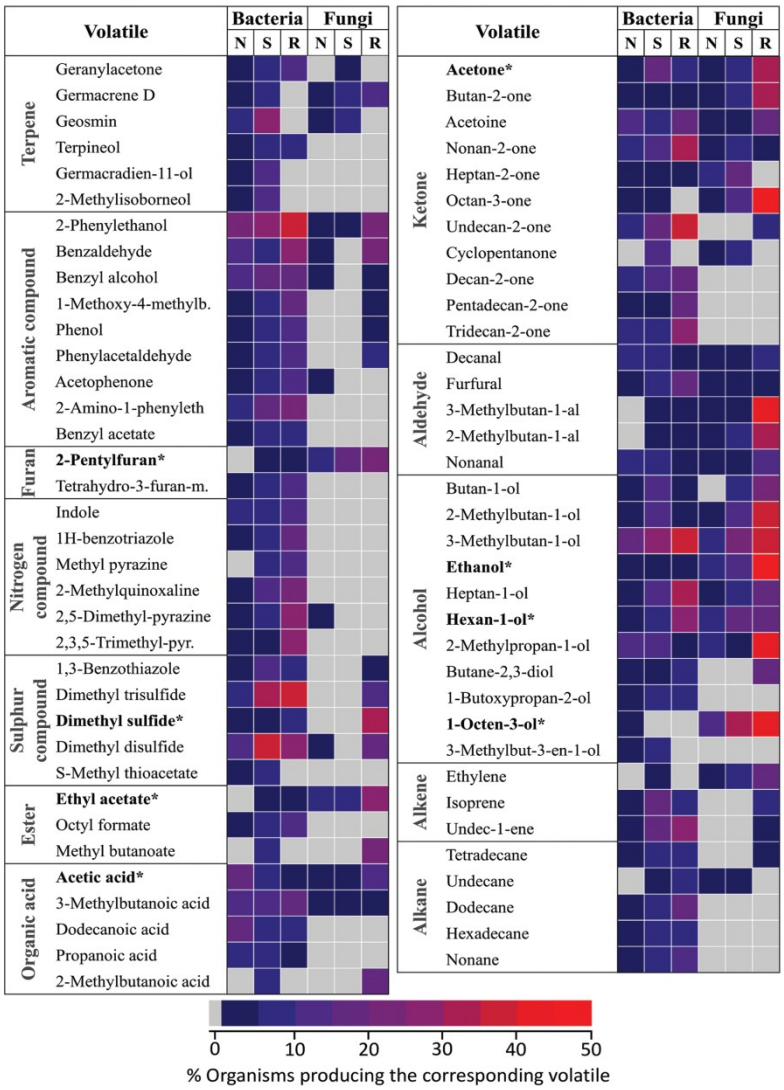

FIGURE 5 | Niche specificity of bacterial and fungal volatiles. Volatiles emitted by at least 10 different organisms from the mVOC database are shown. Microbes were classified based on their lifestyle/habitat as "soil" (120 bacteria, 36 fungi), "rhizosphere" (rhizo: 43 bacteria, 26 fungi) and "other" (157 bacteria, 64 fungi). The color code shows the frequency of each volatile in each group. *These volatiles are also emitted by plant roots.

is well known that media composition, culture conditions or interacting organisms might influence secondary metabolism (Blom et al., 2011; Brakhage and Schroeckh, 2011). Hence if the presence of one volatile in the mVOC database reflects the ability of specific organisms to produce that volatile, its absence does not exclude that it might be produced under natural conditions. The reverse is certainly also true. Overall estimating the total diversity of belowground volatiles will require isolating and characterizing more microbes/plant roots but also analyzing full soil communities under both laboratory and natural conditions. It should be highlighted that profiling volatiles from soil is much more complicated than from any other system. Indeed soil is a highly complex matrix which requires the most advanced instrumentation in terms of resolution and sensitivity (i.e., high resolution MS or proton transfer MS) as well as powerful data processing for harnessing the complexity of its volatilome (Peñuelas et al., 2014; Mancuso et al., 2015).

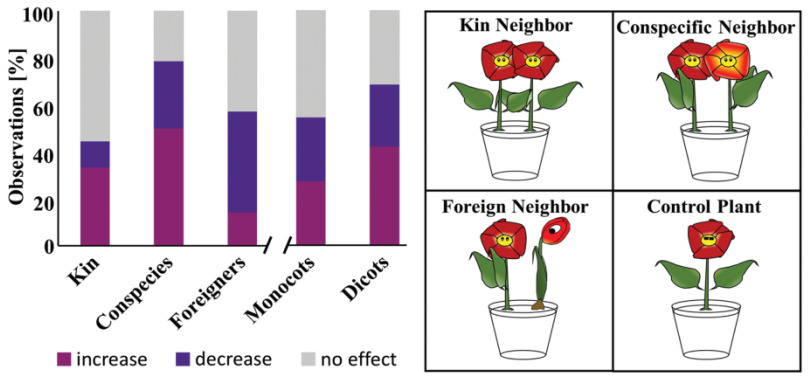

FIGURE 6 | Influence of a neighboring plant on root development. Data presented in a bar chart summarizes the observations of several studies which compared root development of a plant with and without neighbor. The cartoon on the right illustrates the interactions considered here. "Increase, decrease, no effect" refer to the root biomass of a plant with neighbor compared to the one without. Kin: 20 observations in which interacting plants are kins. Conspecies: 27 observations where tested plants are from the same species (but not kins). Foreign: 45 observations where interacting plants belong to different species. Monocots: 28 observations involving monocotyledonous plants. Dicot: 64 observations involving dicotyledonous plants.

Differences and Similarities in Volatile Profiles of Roots, Bacteria, and Fungi

Our analysis highlighted similarities and differences among plant root and belowground microbial volatiles. In terms of similarities, numerous microbes were capable of emitting the same volatiles as plant roots; however, bacteria belonging to the *Firmicutes* (*Bacilli*), to the *Proteobacteria* (β- and γ-*Proteobacteria*) and *Ascomycete* fungi especially distinguished themselves in this regard. Interestingly *Firmicutes* and β- and γ-*Proteobacteria* tend to be dominant root endophytes in rice and sugarcane (Fischer et al., 2011; Sessitsch et al., 2012). Ascomycete fungi also include numerous members which live in close association with plant roots (i.e., truffles forming ectomycorrhizas; Martin et al., 2010). It is therefore tempting to speculate that resemblance in terms of volatile profiles might translate into closer associations between microbes and plant roots. Testing this hypothesis will require characterizing the volatile profiles of numerous plant roots and microbial strains under natural conditions.

How Specific are Belowground Volatile Signals?

Determining how specific volatile signals might be in terms of interactions requires understanding the nature of the interaction and also the habitat in which it takes place. In terms of molecules, terpenoids are not only important volatiles for floral scent (Knudsen et al., 1993, 2006; Pichersky and Gershenzon, 2002), but as illustrated by β-caryophyllene in maize, they might serve as an alarm signal upon attack by root pests (Rasmann et al., 2005). This volatile is also emitted by a bacterium belonging to the *Bacteroidetes* phylum and by some fungi (**Figure 3**; Lemfack et al., 2014). It has additionally been reported from the fungus *Fusarium oxysporum* colonized by ectosymbiotic bacteria, and it is responsible of the growth promoting effect observed in lettuce colonized by the latter fungus (Minerdi et al., 2011). This example illustrates that one volatile might be produced by numerous organisms to a different end. Another terpenoid, geosmin, which is produced by numerous microbes (Lemfack et al., 2014) was also recently reported from beet roots (*Beta vulgaris* sp. *vulgaris*) (Freidig and Goldman, 2014). We had originally not included this compound in our list of plant root volatiles because they were suspicions that it might not have been produced by beet root itself but by microbes colonizing beet roots tissues; however, the data presented by Freidig and Goldman (2014) suggests that this might be otherwise. This highlights that characterizing the volatile profiles of existing plant roots might greatly increase the diversity of plant root volatiles.

Sulfur containing volatiles are also important signals in plant–microbe interactions. Indeed it has recently been demonstrated that dimethyl disulfide produced by Bacillus bacteria naturally colonizing tobacco roots promoted plant growth by enhancing sulfur assimilation (Meldau et al., 2013). Our data highlights that this volatile is predominantly produced by bacteria (essentially soil bacteria) and to a lesser extent by fungi (**Figure 5**). A tempting interpretation might be that numerous soil bacteria might use this volatile for plant growth promotion. Other bacterial volatiles might also serve this purpose, indeed 2,3-butanediol promotes plant growth in Arabidopsis (Ryu et al., 2003). Nevertheless the overall effect of microbial volatiles on plant growth depends on the total volatile blend and cultural conditions of the microbes (Blom et al., 2011; Peñuelas et al., 2014). Therefore understanding the specificity of a signal also requires characterizing the context in which it is emitted as well as the bioactivity of the total volatile blend.

Eight carbon containing volatiles are characteristic of fungi, and its major representative, 1-octen-3-ol is indeed responsible of the typical fungal smell perceived by humans (Wnuk et al., 1983; Mosandl et al., 1986). Our data indicates that 1-octen-3-ol and octan-3-one are predominantly produced by rhizospheric fungi (**Figure 5**). Since numerous of these fungi live in

symbiotic association with plant roots (i.e., truffles), eight carbon-containing volatiles might serve as symbiotic signals to a potential host plant. In terms of biological activity high concentrations of these volatiles have been shown to inhibit seed germination and seedling development in *Arabidopsis* and *Cistus incanus*, a host plant to truffles (Splivallo et al., 2007; Hung et al., 2014). Nevertheless lower concentrations of 1-octen-3-ol was shown to induce plant defense genes in *Arabidopsis* (Kishimoto et al., 2007). These volatile signals might therefore modulate the host–plant fitness, however, how effective this modulation might be in nature remains to be investigated.

Another group of potential signaling molecules are nitrogen-containing volatiles. Interestingly, these volatiles seem essentially produced by bacteria but not by fungi. In relation to their habitat, rhizosphere bacteria were the best producers of these volatiles (**Figure 5**). Since these bacteria include the Rhizobium genus, members of which are able to fix atmospheric nitrogen and hence literally serve as natural fertilizers for legumes when colonizing their roots (Gage, 2004), it can be speculated that nitrogen-containing volatiles are involved in signaling between Rhizobium and legumes. As in the case of dimethyl disulfide (Meldau et al., 2013), nitrogen-containing volatiles might be directly assimilated by legumes for nutritional purposes, however, they might serve other purposes as well. Demonstrating their exact role as signaling agents will first require deciphering their biosynthesis.

The examples above illustrate how specific or unspecific belowground volatile signals might be. The various ecological roles highlighted here and, in some cases, the ability of different organisms to emit the same signals, suggest the existence of complex volatile-based interaction networks. Demonstrating their specificity will require characterizing full networks of interacting organisms but also concentrations-activity ratios as well as the persistence of volatile signals in soil.

Could Root Volatiles be Perceived by Neighboring Plants?

Plants are able to communicate belowground with their neighbors through some unknown signals (Dudley and File, 2007; Bhatt et al., 2011; Fang et al., 2013; Schmid et al., 2013b). Genetic relatedness has recently emerged as an important factor governing belowground root–root interactions. For example roots of rice plants belonging to the same genotype were show to grow toward each other whereas those of different genotypes seem to avoid each other (Fang et al., 2013). Another study involving *Cakile edentula* plants illustrated that plant root allocation is influenced by kinship; indeed the authors observed lower root allocation in kin pairs than stranger pairs (Bhatt et al., 2011). The nature of the signals involved in root–root communication has not yet been fully identified, however, root exudates have been recently suggested as possible candidates (Semchenko et al., 2014). Because volatiles can essentially diffuse further in the soil than root exudates, they might also act as signaling agents in root–root communication. We explored this possibility relying here on indirect evidence. Indeed volatile emission patterns of aboveground plant organs were recently shown to be dependent on kinship, with volatile profiles of kins being more similar to each other than those of plants without kinship (but of the same species) (Karban et al., 2014). Our data demonstrates that how plants respond in terms of root biomass/structure to the presence of a neighboring plant is actually influenced to a certain extent by kinship and genetic relatedness (**Figure 6**). Taken as a whole this suggests that volatile signals might indeed be involved in belowground root–root communications. Demonstrating their exact involvement will require profiling root volatiles as a function of genetic relatedness, identifying the signaling agents, and demonstrating their activity.

Conclusion

The past decade has seen an increasing interest in belowground volatile-based communication among organisms (Kai et al., 2009; Wenke et al., 2010, 2012; Bailly and Weisskopf, 2012; Piechulla and Degenhardt, 2014). Because of the high heterogeneity and large organismic diversity present in the soil, and the potentially humongous diversity of belowground volatiles, it is essential to apply a holistic approach to understand diversity. Such an attempt has been made here essentially relying on the recently published mVOC database of microbial volatiles (Lemfack et al., 2014) and on a limited number of papers describing plant root volatiles. Although our analysis highlights interesting patterns in belowground volatile diversity and distribution, it also cries out for more data. Essentially we might be looking at the tip of the iceberg and estimating total belowground volatile diversity will require characterizing both the emitters and their full volatile spectra. This will be a major challenge considering the huge number of undescribed soil microbes.

Author Contributions

DS and RS wrote the manuscript with input from the other co-authors. ML and BP acquired the data. DS and RS further analyzed and categorized the data and DS created the illustrations. All authors approved the final version of the manuscript.

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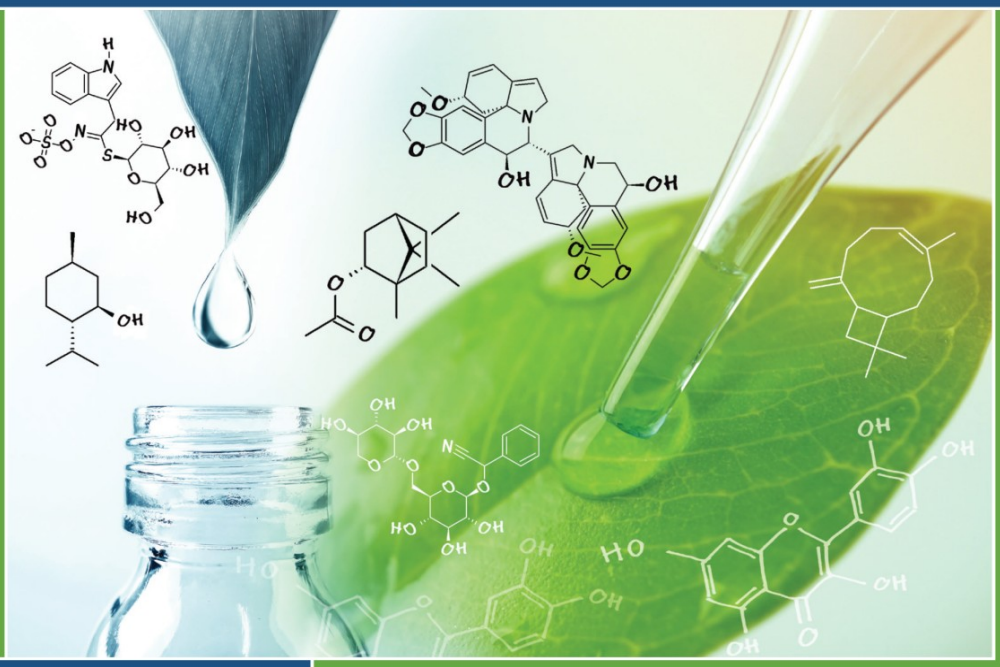
Plant Specialized Metabolism

Genomics, Biochemistry, and Biological Functions



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chapter ten

Microbial volatiles and their biotechnological applications

Birgit Piechulla and Marie Chantal Lemfack

Contents

10.1 Introduction

The capability of the emission of volatile organic compounds (VOCs) is well known for plants and animals. Such volatile compounds are characterized by their molecular weights of less than 300 Da, high vapor pressures, low boiling points, and low polarities. Due to these features, evaporation and distribution into the atmosphere as well as into the air- and water-filled pores below ground are facilitated. These airborne signals exhibit the potential to act as infochemicals for inter- and intraspecific communication in different habitats even over long distances (Kai et al. 2009; Wenke et al. 2010). A comprehensive source of such volatiles is constituted by the microbial world, which has been overlooked in the past. The microbial VOCs (mVOCs) affecting other organisms play a role in plant/fungi-microbe and animal/human-microbe interactions

including microbial pathogens ("the bad") as well as microbes with protecting potential ("the good") (Bailly and Weisskopf 2012; Schenkel et al. 2015; Wenke et al. 2012a). Central questions are: Which volatiles are emitted by microorganisms? Which impact on fitness (health), development and growth do they have on the receiving organism? Are these mVOCs useful for any applications?

At present, headspace analyses of around 490 microbial species resulted in the identification of around 1200 volatiles (for review, see Effmert et al. 2012; Lemfack et al. 2014; Schulz and Dickschat 2007), which are divided into 48 chemical classes dominated by alcohols, alkenes, ketones, and terpenoids (Wenke et al. 2012b). Due to the incredible microbial diversity (10,000 species are known, more than 1 million are expected on Earth) it is foreseen that the actual number of known microbial volatiles represents just the "tip of the iceberg." Furthermore, microorganisms have not been systematically investigated regarding their capabilities of volatile emissions but the present available results indicate that microbes are a good source for novel and unusual volatiles (Lemfack et al. 2014; Von Reuss et al. 2010). The biological and ecological functions of mVOCs are diverse, for example, (i) they play a role in the food chain of the microbial loop since they are assimilated and incorporated into organic matter (bioconversion), (ii) they influence physiological processes in various target organisms (e.g., laccase activity, nitrification, and nitrogen mineralization), (iii) they function as electron acceptors or donors to support metabolic reactions, (iv) they play a role in quorum sensing/quenching, (v) they act as defense compounds against fungi, nematodes/animals and bacteria, (vi) they act as communication signals, or (vii) their functions remain so far elusive (summarized in Effmert et al. 2012; Kai et al. 2009; Wenke et al. 2010 and 2012). Nevertheless, the detailed reactions and adaptations at the physiological, transcriptional, protein and metabolic levels of the target organisms were only recently investigated (Bailly and Weisskopf 2012; Wenke et al. 2012a).

10.2 Most frequently emitted mVOCs

The emission of microbial volatiles is commonly—often unconsciously—recognized; for example, the typical smell of cheese varieties, the aroma of wine, and the characteristic odor of mushrooms derive from microorganisms. Furthermore, the earthy, muddy smell in a wet forest is due to the production of geosmin and other volatiles released by *Streptomyces* species. Microbiologists are trained to recognize indole characteristically emitted by *Escherichia coli* and butyric acid released by *Clostridium* spp. These examples indicate that microorganisms contribute significantly to the odors present in our environment. The most comprehensive summary

Table 10.1 Twenty most cited bacterial volatiles in the mVOC database

	mVOC name	Chemical classification	Number of bacteria emitting the compound
1	2-Phenylethanol	Alcohol	100
2	3-Methylbutan-1-ol	Alcohol	90
3	Dimethyl disulfide	Sulfide	88
4	Dimethyl trisulfide	Sulfide	79
5	Undecan-2-one	Ketone	54
6	Benzyl alcohol	Alcohol	53
7	Geosmin	Terpenoid	52
8	Tetradecanoic acid	Carboxylic acid	52
9	Acetic acid	Carboxylic acid	47
10	2-Aminoacetophenone	Ketone	43
11	Benzaldehyde	Aldehyde	43
12	Nonan-2-one	Ketone	43
13	Acetoin	Ketone	42
14	1-Undecene	Alkene	41
15	2-Methylpropan-1-ol	Alcohol	41
16	3-Methylbutanoic acid	Carboxylic acid	41
17	Dodecanoic acid	Carboxylic acid	41
18	Acetone	Ketone	39
19	1-Heptanol	Alcohol	36

of microbial volatiles is found in the mVOC database (Lemfack et al. 2014). By data mining, the 20 most frequently emitted mVOCs of bacteria (Table 10.1) and fungi (Table 10.2) were determined. The following compounds are significantly more often released by bacteria: (1) 2-phenylethanol, (2) 3-methylbutan-1-ol, (3) dimethyl disulfide, and (4) dimethyl trisulfide; and 1-octen-3-ol is most frequently emitted by fungi. These most abundant mVOCs derive from three different metabolic pathways: (1) the shikimate pathway synthesizing the amino acid phenylalanine (phenylpropanoid biosynthesis), (2) reduction product of isovaleric acid or caprylic acid (fatty acid biosynthesis), and (3) sulfur metabolism. It is interesting to note that alcohols, ketones, and carboxylic acids are the most frequently emitted mVOCs, while terpenoids and pyrazines appear seldom in the VOC spectra of microorganisms.

10.3 Habitats of mVOC producers

Microorganisms appear ubiquitously, in various—even extreme—habitats and ecological niches (Horikoshi et al. 2011). The mVOC database was used to extract the habitats of which volatile emitting microorganisms

Table 10.2 Twenty most cited fungal volatiles in the mVOC database

	mVOC name	Chemical classification	Number of fungi emitting the compound
1	1-Octen-3-ol	Alcohol	62
2	2-Pentylfuran	Furan	44
3	Hexan-1-ol	Alcohol	43
4	2-Pentanol	alcohol	42
5	3-Methylbutanal	Aldehyde	41
6	2-Methylbutanal	Aldehyde	39
7	Hexanal	Aldehyde	38
8	1-Heptanol	Alcohol	37
9	2-Pentanone	Ketone	36
10	2-Ethyl-1-hexanol	Alcohol	34
11	Benzaldehyde	Aldehyde	33
12	Nonanal	Aldehyde	33
13	Decanal	Aldehyde	31
14	Heptanal	Aldehyde	31
15	Styrene	Benzenoid	30
16	1,2,4-Trimethylbenzene	Alkane	29
17	1-Nonanol	Alcohol	29
18	Butan-4-olide	Lactone	29
19	Naphtalene	Alkene	29
20	Pentanal	Aldehyde	29

have been isolated thus far. The microorganisms listed in the mVOC database were obtained from 10 distinct habitats: (1) animals, (2) human/clinical sources, (3) food products, (4) fresh water, (5) humans, (6) marine environment, (7) plants, (8) plant waste, (9) rhizosphere, and (10) soil. Most species listed in the database are of plant sources (70), aquatic environment (66), and from the soil (61). We correlated the VOC profiles of the microorganismal species with the habitat where they originated from (multivariate analysis) (Figure 10.1). No habitat-specific VOC spectra became apparent and it is suspected that it is not the location of isolation, but rather the nutritional supply (growth media) and metabolic capabilities that are relevant for the emission profiles (Fiddaman and Rossall 1993).

10.4 Applications of mVOCs

The biological and ecological functions of the microbial volatiles are very diverse and manifold, and often the biological relevances of mVOCs are not known or understood. Beside this lack of knowledge, mVOCs are

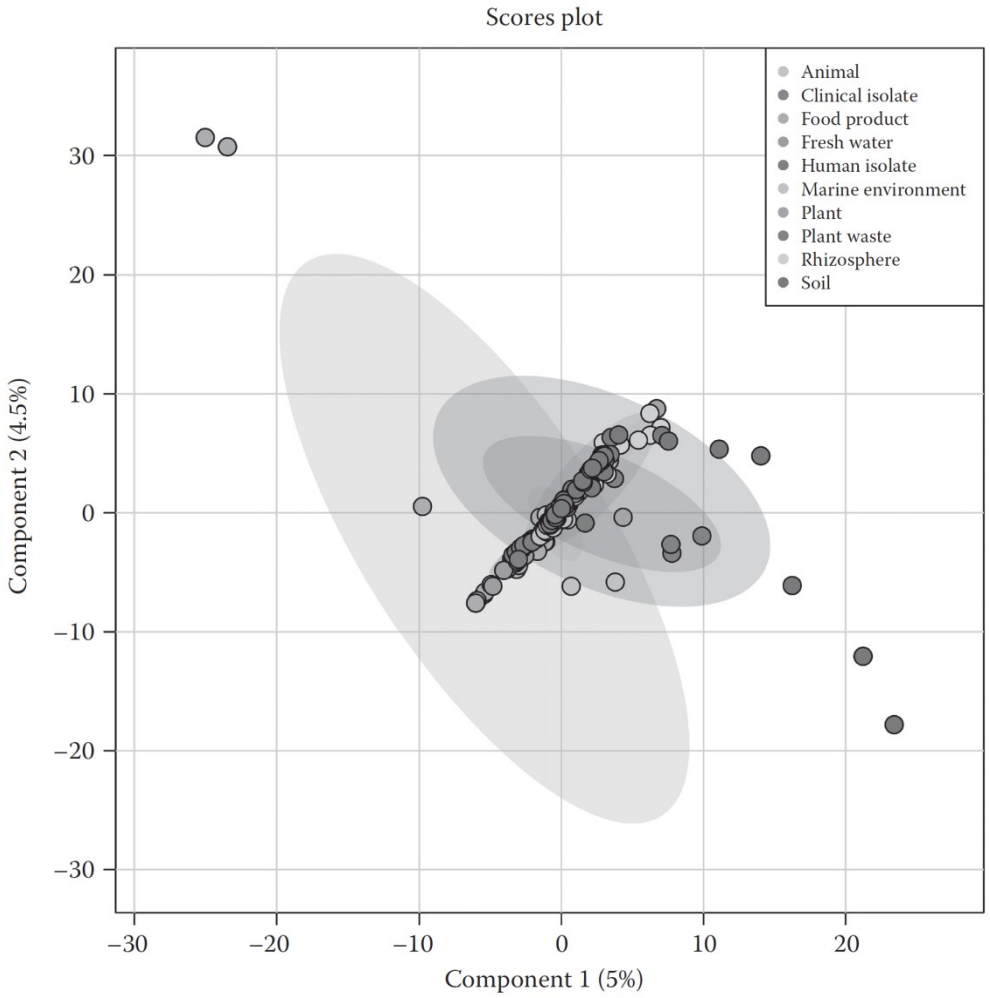

Figure 10.1 (**See color insert.**) Principal component analysis of mVOC-emitting microorganisms of different habitats. Data were extracted from the mVOC database and multivariate analyses were performed using the online comprehensive tool for metabolomic data analysis "MetaboAnalist 3.0." (From Xia, J. et al. 2015. *Nucl. Acid Res.* 43: W251–W257.).

furthermore important cues for applications. Three major areas of implications are addressed: the agricultural, the medical, and the biotechnological application (Figure 10.2).

10.4.1 mVOCs of foodstuff

Flavor involves our perception of sugars, organic acids, and of a diverse group of volatile metabolites produced by multiple metabolic pathways. Although the human nose can distinguish many volatiles, there is a given limitation in the detection and proper description of the relevant smell.

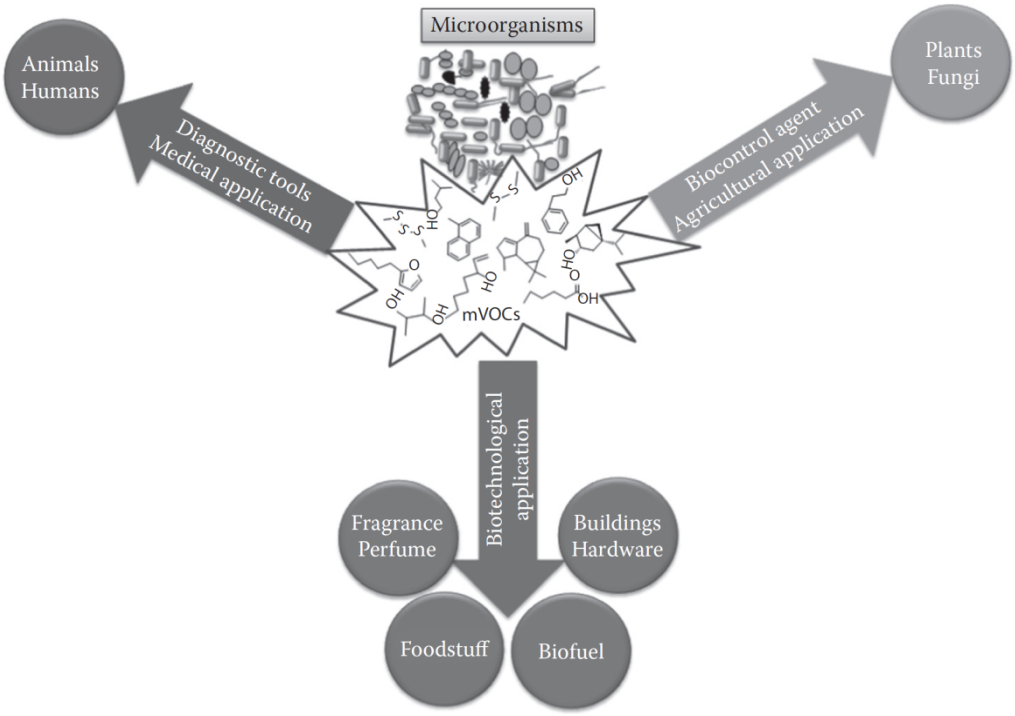

Figure 10.2 Overview of mVOC applications.

Nevertheless, it is still a challenge to improve the flavor of many modern fruit and vegetables. Likewise, the microbial-based volatile aromas of foodstuff such as wine, dairy products, and mushroom (including truffle) are continuously analyzed.

Truffles emit up to 200 volatile metabolites, typical components have a chain length of eight carbon atoms, for example, *trans*-2-octenal, 1-octen-3-ol, and octanol, which are the main components of the mushroom odor (Buzzini et al. 2005; Chitarra et al. 2004) (Table 10.2). The various types of truffles differ in their scent spectra, *Tuber borchii* and *Tuber melanosporum* are distinct to *Tuber indicum* or *Tuber magnatum* (most expensive Piedmont truffle) (Splivallo et al. 2007). New investigations revealed that truffles live in symbiosis with various yeasts, "guest" bacteria, and filamentous fungi, which also contribute to the scent bouquet (Buzzini et al. 2005; Vahdatzadeh et al. 2015). In the case of the white truffle *T. borchii*, it was shown that cyclic sulfur volatiles (thiophene derivatives) actually derive from bacteria inhabiting truffle fruiting bodies (Splivallo et al. 2014). Interestingly, the same thiophene derivatives are also the most important odorants which contribute to human sensed aroma in the latter species (Splivallo and Ebeler 2015). It is believed that truffle fruiting bodies produce their smell to attract mammals and rodents to locate the fungus underground. Actually out of the hundreds of volatiles produced by truffles, only a single one—dimethyl sulfide—has been convincingly

implicated in the attraction of mammals (Talou et al. 1990). The role of other truffle volatiles as a location cue is of great interest for the food industry.

Phenolic content, colour and volatile compounds are the most important *wine* quality attributes. The wine aroma is very much influenced by the bacterial and yeast alcoholic fermentation, when sugars are metabolized into acetic acid as well as ethanol. Acetic esters, ethyl esters and higher alcohols are the dominant aroma compounds, for example, ethyl hexanoate and ethyl octanoate contribute to fruity aromas. Defined bacterial starter cultures are applied to grape juice during wine production. The time point is critical and influences significantly the product outcome. Malolactic fermentation, the decarboxlation of L-malic acid to L-lactic acid is conducted by lactic acid bacteria, for example, *Oenococcus oeni*, results in a slight increase in wine pH and provides microbial stability and positively influences wine aroma and flavour (Abrahamse and Bartowsky 2012). A prolonged or delayed malolactic fermentation increases the risk of spoilage by microorganisms such as *Lactobacillus*, *Pediococcus* and *Brettanomyces* species that may produce, for example, biogenic amines or 4-ethyl-phenol (Curtin et al. 2007; Gerbaux et al. 2009).

Microorganisms play an important role in the development of *dairy product* (cheese, yoghurt, curd, etc.) flavor. For example, more than 100 volatiles, sometimes only present in trace amounts, are found in yoghurt, and most flavor compounds are produced from lipolysis of milkfat and microbial transformation of lactose and citrate (Cheng 2010). Most important for the aroma are acetaldehyde, diacetyl, acetoin, acetone, and 2-butanone, while off-flavor compounds appear during extended shelf-lifes due to lipid oxidation. Since flavor is a very important characteristic from the consumer's point of view, new strains isolated from dairy and nondiary environments are tested for their production of odor-active volatile compounds to modulate dairy product flavors (Pogacic et al. 2015). Multivariate analyses are used to evaluate mVOC profiles of typical milk fermenting bacteria such as *Leuconostoc lactis*, *Lactobacillus* spp., *Brachybacterium* spp., *Brevibacterium* sp., and *Propionibacterium* sp. The survey identified 52 mVOCs and certain VOCs such as ethyl esters, sulfur compounds, branched chain alcohols and acids, and diacetyl as well as related carbonyl compounds turned out to be characteristic for each bacterial species (Pogacic et al. 2015). It was also possible to differentiate mVOC profiles of, for example, the New Zealand and the Italian parmesan cheese based on butanoic acid, phenylacetaldehyde, ethyl butyrate, acetaldehyde and methylbutanals (Langford et al. 2012). Thus, microbial volatile aroma compounds are important tools to assess dairy product quality and flavor although much work is still necessary to understand the complete formation of aromas and flavors. Qualitative and quantitative analyses are surely a first step to achieve this goal, but the volatile compound-matrix

interaction, release mechanism of VOCs and synergistic actions need to be addressed to assess their aroma contributions (Cheng 2010).

The most well-known *"off-flavor"* volatile geosmin is emitted by soil bacteria such as *Streptomyces* spp., *Anabena* spp., *Oscillatoria* spp., myxobacteria, soil-dwelling, aquatic and airborne fungi. Geosmin has the characteristic earthy and moldy odor and is recognized as a volatile of contaminated food, wine and water (Darriet et al. 2000). The repugnant smell of rotting organic matter results from the release of bacterial and fungal volatiles, consequently mVOCs are good markers for spoiled foodstuff, for example, meat, bread, vegetables and fish. mVOCs used as indicators in food industry will be exemplified here for fish processing. The European sea bass with its white flesh and low fat content is a popular farmed fish. It is very perishable due to microbial spoilage (Gram and Hass 1996). Typical spoilage microorganisms reach high population densities and produce several metabolites (CSIs: chemical spoilage indices), which are responsible for the off-odor resulting in their organoleptic rejection (in Parlapani et al. 2015). During fish deterioration, the production of microbial volatile compounds such as trimethylamine, dimethylamine, and ammonia is measured with a hydrogel-pH-electrode based near-field passive volatile sensor (Bhadra et al. 2015). Other mVOCs have also been studied as potential indicators for CSIs for spoilage/freshness evaluation and became important measures for food quality when the levels vary between the initial and rejection day of seafood. Most of the 40 mVOCs determined on sea bass appeared sporadically or fluctuated, while, for example, 3-methyl-1-butanol, 2-methyl-1-butanol and the ethyl esters acetate, propionate and isobutyrate increased during storage. Various other mVOCs have been reported as metabolites released by *Pseudomonas* spp., *Schewanella* spp., *Enterobacteriaceae*, and *Brochothrix thermosophacta* during fish and/or meat spoilage and specifically ethyl esters were related to *Pseudomonas* activity (Casaburi et al. 2014). Indeed, esters were found only in gutted sea bass stored under air where the aerobic conditions enhance the growth of pseudomonads (Parlapani et al. 2015), while ethyl acetate increased in cod (Olafsdottir et al. 2005) and pangasious filets (Noseda et al. 2012). Acetic acid was mainly attributed to the growth of *B. thermosophacta*. These examples indicate that the characteristic mVOC profiles (= fingerprints) depend on storage conditions (air, vaccum, temperature), fish or meat batches, and on the microorganisms, which preferentially colonize the fish species or the kind of meat. The development of biosensors based on mVOCs is a reachable goal assessing freshness and shelf-life of foodstuff.

10.4.2 mVOCs as indicators of damp buildings and other hardware

In the 1990s, mVOCs were used as indicators for indoor air environment (Bayer and Crow 1994; Wessen et al. 1995). With this technique, it was

possible to detect hidden microbial growth behind interior surfaces without destructing the building because it was assumed that gases (mVOCs) may enter the indoor air more easily than fungal or bacterial spores (Lorenz et al. 2002; Wessen et al. 1999). The term "sick building syndrome" (SBS) was coined in the last decade to refer to a set of symptoms that are experienced by the occupants of a building with poor air quality (Polizzi et al. 2012). Buildings with moisture and mold damage were considered risky for health, particular for eyes and the upper respiratory tract (cough and wheeze). Korpi et al. (2009) summarized 96 typical "indoor" mVOCs, of which 15 compounds were toxicologically evaluated (e.g., inhalation studies, lowest administered doses). From the human experimental exposure studies it turned out that symptoms of irritation appeared at mVOC concentrations several orders of magnitude higher than those measured indoors and it was concluded that mVOC concentrations are too low to provoke a nuisance effect on the building occupants. However, a drawback of the present analysis is that the toxicological database is poor, and there may be more potent compounds and endpoints yet not evaluated. Furthermore, in the environment mVOCs may come from various sources, such as building materials, human activities, traffic, foodstuff, smoking and may overlap and act additively in mixtures. The majority of mVOC producers present on buildings are fungi, for example, *Alternaria, Aspergillus, Botrytis, Candida, Fusarium, Penicillium, Trichoderma*, but also bacteria *Streptomyces* and *Pseudomonas* appear frequently (Claeson et al. 2002; Korpi et al. 1998). The main mVOCs produced on building materials were 3-methyl-1-butanol, 1-pentanol, 1-hexanol and 1-octen-3-ol, and Korpi et al. (1998) concluded that no single VOC is a reliable indicator for biocontamination. In contrast Bennett and Inamdar (2015) reported on some mVOCs that have toxic properties. Experimental tests with tissue cultures and *Drosophila melanogaster* have shown that many single mVOCs as well as mixtures emitted by fungi have toxic effects. Subsequently, they are referred to mycotoxins. Inamdar et al. (2010, 2013) tested low vapor concentrations of C-8 compounds, including 1-octen-3-ol, and showed toxicity to larvae and adult flies, selectively affecting dopaminergic neurons in adult *Drosophila* brains and induced Parkinson like behavioral alterations. The toxicity data on 1-octen-3-ol are of particular concern, because this fungal VOC appears ubiquitously (it is the most prominantly released fungal mVOC, Table 10.2) and is largely responsible for the musty odor commonly associated with mold-contaminated damp indoor spaces (Bennet and Inamdar 2015). In accordance, the results of a recent study concerning 6-pentyl-2-pyrone production by *Trichoderma atroviridae* growing on buildings showing that fungi can support SBS symptoms by irritating and damaging mucosal membranes (Polizzi et al. 2011), even when the production of fungal VOCs varies and is suboptimal due to the dependence on temperature and humidity (Polizzi et al. 2012).

Microbial biofilms are also formed on much other hardware, for example, clinical tubings, hulks, and air conditioners of houses and automobiles, which may result in malodors. Alpha-proteobacteria, methylobacteria, Shingomonadales, Burkholderiales, Bacillales, *Alcanovorax* spp., and *Stenotrophomonas* spp. were found on contaminated heat exchanger fins of evaporators from cars, which produced di-, tri-, and multiple sulfides, acetylthiazole, aromatic compounds and diverse pyrazines (Diekmann et al. 2013). Interestingly, a close relationship of the VOC profile and microbial community to the climate and air quality where the car was operating (European, American, Arabic, and Asian) was determined.

10.4.3 mVOCs for the perfume industry

Volatile organic compounds are predestinated and prestige flavor, fragrance, and aroma compounds. The perfume industry has an increasing demand for new VOCs. Although traditionally plants were the sources for aromas and fragrances, many other sources including microorganisms are presently tapped (Rutkin 2015).

10.4.4 mVOCs as the next generation biofuel

The limitations of fossil fuels are foreseen and contrast steadily increasing fuel requirements (reviewed in Rude and Schirmer 2009). Furthermore to combat climate change and to reach the goal of energy independence, search for alternative and renewable energy sources is ongoing. Three routes to convert such resources into energy-rich, fuel-like molecules or fuel precursors are considered: (i) photosynthesis related production by plants, alga and cyanobacteria, (ii) fermentative and nonfermentative production by heterotrophic microorganisms, and (iii) chemical conversion of biomass. Biofuel platforms based on food and nonfood biomass conversion have several limitations including the energy output per land area, the compatibility with current fuel infrastructures, and insufficient capacity the meet Renewable Fuel Standard (RFS) (Wang et al. 2015). mVOCs are considered as potential alternatives of biofuels from renewable resources. Microbial fuels are fermentative short–chain alcohols (e.g., ethanol and butanol), nonfermentative short-chain alcohols (1-propanol, 1-butanol, isoforms and derivatives of butanol, pentanol, and 1-hexanol), fatty acid-derived hydrocarbons (fatty acid alcohols of C12 to C18 are useful for fuels, while very-long-chain fatty acid alcohols C24–C26 are unsuitable), and isoprenoid-derived hydrocarbons. To overcome the disadvantages of ethanol, fuel research enquired the production of isoprenoid derived biofuels. Sesquiterpenes (e.g., farnesol, farnesene, and farnesane bisabolene) are being developed as precursors for fuel and monoterpenes (e.g., α-pinene, camphene, limonene, and sabinene and terpinene) are discussed

as potential next generation jet fuel components (reviewed in Gupta and Phulara 2015; Rude and Schirmer 2009). Isoprenoides are considered as good diesel alternatives because of their low hygroscopy, high energy content and good fluidity at low temperatures. Limitations are the low yields, toxicity and their stereochemical complexities. Presently attempts are undertaken to use genetically engineered microbes that produce a variety of infrastructure-compatible drop-in fuel molecules and to boost up terpene synthesis. In recent years, particularly *Escherichia coli* and *Saccharomyces cerevisiae* as well as less commonly used microbial strains were used as hosts to tune expression of endogenous enzymes of the isoprenoid pathway or to introduce heterologous enzymes. Several obstacles have been addressed and overcome, and advances in isoprenoid-based biofuels have been made using synthetic biology tools, for example, modulating the MEP pathway because it is stoichiometrically more competent than the MVA pathway, introducing the second terpene pathway into the microorganism, reprogramming metabolic nodes of pathways via mutations of transcription factors, improving terpene synthase catalysis, and installing efficient storage and excretion strategies are essential keys for progress (summarized in Gupta and Phulara 2015). Cyanobacteria, due to their fast growth, high photosynthetic rates, ability to grow in nonarable areas, availability of genome sequences, and production from CO_2 and solar energy make them a useful platform for biofuel production (Gupta and Phulara 2015; Wang et al. 2015). Although isoprenoid-based biofuel titers have been significantly improved in microorganisms in recent years, they still do not match with those of ethanol.

10.4.5 mVOCs as biocontrol agents in agriculture

Microbiota are attracted by suitable microenvironments in the soil to colonize and create microecosystems. Consequently, the "networking" communities are characterized by mutualism, commensalism, cooperation, antagonism, competition, and coexistence. In this arena, interactions between bacteria and fungi could have a positive or negative impact on third parties, which is useful if the weakened party is a pathogen and the strengthened party is a valuable member of the community (plant growth–promoting bacteria or fungi). An example of such a kind was recently shown (Kottb et al. 2015). The dominant volatile 6-pentyl-pyrone (coconut-like aroma, 6PP) of *Trichoderma asperellum* was perceived as a stress compound by *A. thaliana* and subsequently initiated multilayered defence adaptations including morphological and physiological alterations as well as activation of signaling cascades to withstand this environmental influence. Most noticeable is that *A. thaliana* preexposed to 6PP showed significantly reduced symptoms when challenged with *Botrytis cinerea* and *Alternaria brassicicola*, indicating that defense-activated plants

subsequently became more resistant to pathogen attack. Together, these results support that products that are based on *Trichoderma* volatiles have the potential of being a useful biocontrol agent in agriculture.

Another example is a GFP-tagged *Bacillus subtilis* strain which was able to successfully suppress cucumber wilt by confining growth of *Fusarium oxysporum* f. sp. *cucumerinum* by colonizing the root and persisting on the rhizoplane (Cao et al. 2011). The authors proposed antibiosis as one mode of action caused by diffusible agents. Other experiments with a *B. subtilis* strain isolated from the rhizosphere of wheat and soybean showed bacterial volatiles being involved in the biocontrol of *Botrytis mali* and *Phytophthera sojae*, respectively (Jamalizadeh et al. 2010; Sharifi et al. 2002).

Many *Bacillus* species have been reported to synthesize plant growth promoting VOCs. Ryu et al. (2004) reported that 2,3-butandiol and acetoin emitted by *Bacillus subtilis* GB013 and *Bacillus amyloliquefaciens* IN937a triggered induced systemic resistance against *Erwinia carotovora* in *Arabidopsis thaliana*. Volatiles of *Bacillus badius* M12 were documented to possess organogenetic potential on callus cultures of *Sesamum indicum* and introduce antioxidative activity in tobacco cultures (Gopinath et al. 2015).

Microorganisms of the soil and rhizosphere are able to protect plants from infections by specific root pathogens, a phenomenon called disease-suppression of soils (Hornby 1983). Beside other antagonistic modes of actions, only in the last decade it appeared that mVOCs play a key role in this pathogen inhibition (Insam and Seewald 2010; McNeal and Herbert 2009). The biological activity of such mVOCs might be an alternative regarding the usage of the methyl bromide for fumigation of soils infected by soil-borne fungal pathogens; in fact dimethyl disulfide, which is frequently emitted by bacteria (Table 10.1), is already used as a novel soil fumigant PALADIN®. Fungistasis results from the presence of mVOCs, for example, blocking germination of spores and inhibiting mycel growth (summarized in Kai et al. 2009). Minerdi et al. (2009) also showed that virulence genes are repressed in *Fusarium oxysporum*. Only a few studies indentified single compounds being responsible for the antifungal activity. Fernando et al. (2005) and Cordovez et al. (2015) demonstrated that six VOCs (cyclohexanal, decanal, 2ethyl-1-hexanol, nonanal, benzothiazole, and dimethyl trisulfide) of *Pseudomonas* and 1,3,5-trichloro-2-methoxy benzene and methyl 2-methylpentanoate of *Streptomyces* species possessed antifungal activity. However, the concentrations tested were quite high and the ecologically relevant doses remain to be determined.

mVOCs are also nematicidial. Gu et al. (2007) showed that mVOCs of bacilli isolates reduced movement of the nematodes *Panagrellus redivivus* and *Bursaphelenchus xylophilus.* Microbial volatiles also influence the tritrophic interactions comprising bacteria, fungi, and nematodes. *Paenibacillus polymyxa* and *Paenibacillus lentimorbus* exhibited strong

antifungal activities, thereby interfering with the nematode-fungus interaction (*Meloidogyne incognita—Fusarium oxysporum),* which significantly reduced nematode infestation of tomato plants (Son et al. 2009). Additionally, soil bacteria, including one rhizobacterial strain, enhanced the nematophagous activity of the nematode-trapping fungus *Arthrobotrys oligospora* by increasing trap formation and predaceous activity (Duponnois et al. 1998). There are also numerous instances of mVOCs associated with insect feeding behavior, but some mVOCs are also powerful repellants (summarized in Davis et al. 2013). In some ecosystems, bacterial and fungal volatiles incite insect aggregations, or mVOCs can resemble sexual pheromones that elicit mating and oviposition behaviors from responding insects. An interesting example, which is considered a novel approach to control aphids in the field and greenhouse, is due to the release of volatiles by *Staphylococcus sciuri* allowing to locate the prey in the tritrophic aphid–bacteria hoverfly interaction (Leroy et al. 2010).

Considerable progress has been made in understanding the functions of mVOCs in plants; however, implanting this knowledge under field conditions remains in its infancy (summarized in Kanchiswamy et al. 2015). Field trials are needed to prove the value of mVOCs, but there is a realistic chance to develop new sustainable, cheaper, efficient, effective, and eco-friendly alternatives to pesticides and fertilizers (Kanchiswamy et al. 2015).

10.4.6 mVOCs for chemotyping and diagnostic tools

In addition to considering the biological and ecological roles of mVOCs being important for the homeostasis (fitness and health) of humans and plants, these compounds can be used as noninvasive markers. Individual mVOCs as well as clusters of volatiles are useful for phenotyping fungi (Müller et al. 2013) and bacteria (Peñuelas et al. 2014). Even phylogenetically closely related but phenotypically different species of *Streptomyces* isolates or *Clostridum difficile* ribotypes could be differentiated (Cordovez et al. 2015; Kuppusami et al. 2015). Noninvasive markers are particularly demanded and already used as medical diagnostic tools, for example, for the recognition of methyl nicotinate as an indicator for tuberculosis caused by *Mycobacterium tuberculosis* (Syhre and Chambers 2008; Mgode et al. 2012). *Pseudomonas aeruginosa* infections are associated with declining lung function in cystic fibrosis and high mortality rates. 2-aminoacetophenone is a small molecule and an intermediate of the quinazoline biosynthesis and was shown to be significantly higher in *P. aeruginosa* colonized subjects than control patients and is therefore a promising breath biomarker (Scott-Thomas et al. 2010). A noninvasive VOC-based detection was proposed as an alternative technique suitable for surveillance and as diagnostic tool applicable for urological infections by *Proteus* spp. (Aarthi et al.

2014). These examples stand for a number of other cases described in the literature; this chapter was not initiated to present a comprehensive review of this topic.

10.5 Conclusion

Microbial VOCs have been overlooked in the past, but due to the ubiquitous appearance of microorganisms and their broad metabolic capabilities they have to be considered to understand organismal interactions in ecosystems. Besides these semiochemical functions and biological relevances, mVOCs offer chances to provide sustainable and eco-friendly alternatives to pesticides and fertilizers in the agriculture and the environment, and noninvasive diagnostic tools for animal and human health and fitness. mVOCs are already used in biotechnological processes to control, for example, foodstuff, buildings, and other hardware. It is expected that techniques with better sensitivity and wider applications will be used routinely to make our lives safer. Furthermore, we expect to find novel (lead) structures of natural products that are good sources for new applications.

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Novel volatiles of skin-borne bacteria inhibit the growth of Gram-positive bacteria and affect quorum-sensing controlled phenotypes of Gram-negative bacteria

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ABSTRACT

The skin microbiota is import for body protection. Here we present the first comprehensive analysis of the volatile organic compound (VOC) profiles of typical skin-resident corynebacterial and staphylococcal species. The VOC profile of *Staphylococcus schleiferi* DSMZ 4807 was of particular interest as it is dominated by two compounds, 3-(phenylamino)butan-2-one and 3-(phenylimino)butan-2-one (schleiferon A and B, respectively). Neither of these has previously been reported from natural sources. Schleiferon A and B inhibited the growth of various Gram-positive species and affected two quorum-sensing-dependent phenotypes – prodigiosin accumulation and bioluminescence – of Gram-negative bacteria. Both compounds were found to inhibit the expression of prodigiosin biosynthetic genes and stimulate the expression of prodigiosin regulatory genes *pigP* and *pigS*. This study demonstrates that the volatile schleiferons A and B emitted by the skin bacterium *S. schleiferi* modulate differentially and specifically its interactions with members of diverse bacterial communities. A network of VOC-mediated interspecies interactions and communications must be considered in the establishment of the (skin) microbiome and both compounds are interesting candidates for further investigations to better understand how VOCs emitted by skin bacteria influence and modulate the local microbiota and determine whether they are relevant to antibiotic and anti-virulence therapies.

Introduction

The skin is the body's most extensive organ and serves as a barrier between the internal and external environments. It protects the organism from harmful agents, excessive loss of water and microbial assault [37,49,59]. Since the skin is in permanent contact with the external environment, it is heavily colonized by diverse species of microorganisms, collectively known as the skin microbiota, which plays a key role in health and disease [55]. Thus, over the past decade, analysis of the function of the skin microbiome has become a topic of considerable interest. It is well known that the skin microbiota includes fungi, viruses and mites, but bacteria are generally dominant. Based on the analysis of 16S rRNA, it has been shown that approximately 1000 species of bacteria can be found on the human skin [21,22]. They belong to 19 phyla, of which *Actinobacteria*, *Firmicutes* and *Bacteriodetes* predominate. Although it was recently shown that the host genome has an impact on the skin microbiota, little is known about how its composition is controlled [62]. Nevertheless, most skin-resident bacteria are non-pathogenic commensals, and it has become apparent that some species are beneficial to their host. The microbiota is made up of complex dynamic communities of microorganisms, which, for example, interact with immune cells to modulate the skin immune system by priming T-cells to recognize non-self antigens for appropriate immune responses [6,40,53]. By colonizing the skin, the normal bacterial population can also compete with and eliminate pathogens through surface occupation. They can inter-

Abbreviations: mVOC, microbial volatile organic compound; QS, quorum sensing.

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act with other microorganisms by secreting various metabolites, including toxins and antibiotics, such as the anti-microbial peptides bacteriocins [5]. For example, strains of *Staphylococcus warneri* can produce warnerin, which inhibits the growth of a large number of Gram-positive and Gram-negative bacteria [48]. In the same way, *Staphylococcus epidermidis* and *Staphylococcus gallinarum* secrete the lantibiotics epidermin and gallidermin, respectively, which belong to a class of bacteriocins that inhibit other Gram-positive bacteria [18,30,57]. Bacteria on the skin can also produce compounds of low molecular weight, which are often volatile due to their high vapor pressure and low boiling point. These are collectively termed microbial volatile organic compounds (mVOCs) [51,58,60,68]. The potential effects of mVOCs on the skin microbiota have largely been overlooked. Nevertheless, it is known that the secretions produced by the skin glands are usually odorless, bacterial metabolism can transform these substances into odoriferous volatile compounds [25,26,60,67,77]. Thus, species of *Corynebacterium* degrade various precursor compounds found in sweat into short branched-chain fatty acids, such as (*E*)-3-methyl-2-hexenoic acid, which is the primary contributor to the typical axillary odor and is a key scented volatile [41,80]. In addition, *S. epidermidis* degrades leucine present in the sweat to produce 3-methylbutanoic acid, which is the major component of foot odor [1]. Other studies have shown that volatile aliphatic carboxylic acids and dimethyl disulfide produced by the skin microbiota are the principal cues that mosquitoes use to locate humans [50,68,70].

The spectrum and diversity of known mVOCs were recently summarized in the mVOC database [34], which lists more than 1200 mVOCs that have been described so far. Volatile profiles of individual microorganisms often reveal compounds that are completely new to nature. One such species is the rhizobacterium *Serratia plymuthica* 4Rx13, which releases more than 100 volatiles, including the novel compound sodorifen that has a unique and unusual structure [71,74]. Moreover, some bacteria produce compounds of particular interest, which possess antibiotic, antifungal, nematicidal or plant-growth-promoting properties and/or potentially function as signal molecules in communication within microbial communities [2,9,14,15,16,24,28,52,56,64].

It is likely that the skin represents a habitat characterized by strong interactions between its normal microbial residents and/or with other environmental microorganisms. However, while little is known about how these communities maintain their stability on the skin, it is tempting to speculate that mVOCs might play important roles in microbial interactions and defenses. In order to gain a better understanding of the role of these mVOCs, the VOC profiles of different bacterial species naturally found on the skin were first analyzed. The effects of bacterial volatiles on other bacteria were then studied, and it was found that volatiles produced by *Staphylococcus schleiferi* DSMZ 4807 inhibited the growth of Gram-positive bacteria and affected the phenotypes of Gram-negative bacteria that are controlled by quorum sensing. Among the volatiles released by *S. schleiferi* isolates, amino/imino ketones were identified that had never been reported from any other organism. Therefore, the structures, biological effects and modes of action of these substances, which were designated schleiferons A and B, on the growth of Gram-positive and Gram-negative bacteria are described.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study, and their origins, are summarized in Table S3. Brain heart infusion medium (BHI) (Roth, Germany) was used as the culture medium for all strains except *Serratia marcescens* V11649 and *S. plymuthica* AS9, which were grown in peptone glycerol broth (5 g peptone, 2 g K_2HPO_4, 10 mL glycerol per L of medium), and *Vibrio harveyi* DSMZ 6904, which was grown in Basic medium (10 g tryptone, 5 g yeast extract, 1.5 mL of 50% glycerol, 20 g NaCl, 1 g $MgSO_4$, 6 g Tris-HCl, pH 7.5 with HCl per L of medium). Bacterial stocks were prepared by adding glycerol (final concentration 25%) to an overnight culture, and stored at −70 °C.

Collection and analysis of mVOCs

A single colony of each strain was transferred from a Petri dish to 8 mL of BHI and incubated at 30 °C under agitation (170 rpm) for 24 h in order to obtain a fresh, pure pre-culture. The cell density of each pre-culture was measured at OD_{600} (0.05–1) and an aliquot was transferred into a modified 250-mL conical flask containing 100 mL of culture medium (final OD_{600} of 0.005). The culture was set up in a closed-airflow VOC collection system ([27], modified) connected to a pump (Gardner Denver Thomas GmbH, Memmingen, Germany) and incubated at 30 °C under agitation (Fig. S8). Charcoal-purified air, sterilized by passage through a wad of cotton wool, was introduced into the conical flask containing the bacterial culture at a constant flow rate (500 mL min^{-1}). After passing over the bacterial culture, the volatile-enriched air was further funneled into a trap containing 30 mg of adsorbent matrix (Porapak™, Waters, Eschborn, Germany). After a defined incubation period (see specific figure or figure legend), the volatiles were eluted from the matrix with 300 μL of dichloromethane. Nonyl acetate (10 μL; equivalent to a final concentration of 5 ng μL^{-1} in the eluate) was added as an internal standard. Samples were analyzed using a Shimadzu GC/MS QP 5000 (equipped with a 60 m × 0.25 mm × 0.25 μm DB5-MS column). Using a CTC autosampler, 1 μL of the eluate was injected directly (without flow splitting) at 200 °C with a sampling time of 2 min. Helium was used as the carrier gas. Mass spectra were obtained using the scan mode. Compounds were identified by comparing their retention times and mass spectra with those of the authentic compounds or with those available in the National Institute of Standards and Technology (NIST) 107 library (version 1998). As a control experiment, the volatiles emitted from the media were determined at respective time intervals and were always subtracted from the bacterial volatile profiles and did not appear in the analyses. The media volatile profiles never showed schleiferon A or B. Furthermore, the volatiles of the media continuously decreased from interval to interval, indicating that at later stages during bacterial growth when schleiferon became dominant the volatiles of the media became minor compounds. To ensure that the volatiles analyzed were derived from the bacteria, two control experiments were performed: (i) the supernatant of the overnight culture was sterile filtered and incubated, and (ii) the supernatant was heated to inactivate enzymes, sterile filtered and incubated. Schleiferons A and B were not detected in either experiment, and only volatiles from the media were present (data not shown). The quantities of schleiferons A and B produced were calculated based on the internal standard. Schleiferons A and B were synthesized from 2-phenylethylamine and acetoin (Schulz et al., in preparation).

Effects of S. schleiferi *VOCs on other microorganisms*

Dual culture experiments were performed using 96-well microtiter plates (Fig. S3) or bipartite Petri dishes. A total of 40 wells of each microtiter plate were filled with 200 μL of an *S. schleiferi* DSMZ 4807 culture (OD_{600} 0.005) and another 40 wells were inoculated with 200 μL of the test bacterium culture (*Staphylococcus sciuri* V405, *Staphylococcus saccharolyticus* B5709, *S. epidermidis* RP62A, *Staphylococcus haemolyticus* CCM 2729, *Enterococcus faecalis* ATCC 51299, *Escherichia coli* DH5α, *Pseudomonas fluorescens* V12141, *S. marcescens* V11649 and *Salmonella enterica* RV4) of

OD_{600} (0.005). The microtiter plate was incubated at 30 °C in a microtiter plate reader (SpectraMax M2, MWG-Biotech, Ebersberg, Germany) for 72 or 96 h. Every 30 min the plate was shaken for 15 s and the cell density was measured. In the control experiment, the plate was filled with 200 μL of BHI instead of the *S. schleiferi* DSMZ 4807 culture.

For dual culture in bipartite Petri dishes, one compartment of the plate was filled with 10 mL of a 96 h culture of *S. schleiferi* or *S. warneri* (control) and the other was inoculated with 10 mL of the test bacterium. In a second control experiment, 10 mL of BHI instead of the *S. schleiferi*/*S. warneri* culture were added to one compartment of the Petri dish. The plate was incubated for 24 h at 30 °C, and the growth of the test bacteria was monitored by determining living cell numbers (CFU) and cell densities (OD_{600}).

For the liquid dual culture system (Fig. S9), *S. schleiferi* DSMZ 4807 and *S. warneri* CCM 2730 cultures (OD_{600} 0.005) were grown at 30 °C under agitation (170 rpm). After 48 h or 96 h incubation, 100 mL of each culture was transferred into a 250-mL modified conical flask (Fig. S9). In this set-up, charcoal-purified and sterilized air was pumped as described above into a bacterial culture flask (*S. schleiferi* or *S. warneri* as a control) at a constant rate of 700 mL min^{-1}, and the volatile-enriched air was allowed to pass through an outlet of the first flask into a second flask containing *S. marcescens* V11649 or *V. harveyi* DSMZ 6904 (150 mL culture OD_{600} 0.005). After 24 h, the growth of *S. marcescens* or *V. harveyi* was monitored by determining living cell numbers (CFU) and cell densities (OD_{600}).

Effects of synthetic schleiferons A and B on quorum-sensing-dependent phenotypes of Gram-negative bacteria

Overnight cultures of *S. marcescens* V11649, *S. plymuthica* AS9 or *V. harveyi* DSMZ 6904 were incubated at 30 °C under agitation (170 rpm) (20 °C for *S. plymuthica*). An aliquot of this culture (OD_{600} 0.5–1) was then transferred into a 25-mL Erlenmeyer flask or to one side of a bipartite Petri dish, both containing 4 mL of culture medium (final OD of 0.005). Various concentrations of schleiferon A or B solved in DMSO were added either directly into the bacterial culture or onto a 6 mm Whatman filter paper disk, which was placed in the other side of the Petri dish. The bacterial culture was incubated under agitation in the Erlenmeyer flask, and the Petri dish or Erlenmeyer flask were incubated at 30 °C (20 °C for *S. plymuthica*). After 20 h the cell density was determined. Prodigiosin was extracted from *S. marcescens* and *S. plymuthica* cultures and quantified as described below. *V. harveyi* bioluminescence was determined using the Stella Image Reader (Raytest, Straubenhardt, Germany) and quantified with Aida Image Analyser software. In control experiments, DMSO (dimethyl sulfoxide) was used instead of schleiferons A and B.

Extraction of prodigiosin

S. marcescens or *S. plymuthica* cultures were centrifuged at 10,000 × *g* for 5 min, the supernatant was discarded and the pellet resuspended in 96% ethanol acidified with 5% HCl. After centrifugation at 10,000 × *g* for 5 min, prodigiosin was quantified by measuring its absorption in the supernatant at 534 nm.

Determination of minimum inhibitory concentrations (MICs) for schleiferons A and B

MIC determinations were carried out on various Gram-positive and Gram-negative test species in 25-mL Erlenmeyer flasks (each containing 4 mL of culture medium), which had been inoculated (to OD_{600} = 0.005) with aliquots of cultures grown to OD_{600} 0.5–1 overnight at 30 °C and 170 rpm. Different concentrations of schleiferon A or B diluted with DMSO were added to the test cultures and incubated under agitation at 37 °C. After 20 h, the CFU and cell densities (OD_{600}) were determined. DMSO was used instead of schleiferon A or B in control experiments.

Preparation of RNA probes

Dioxygenin-labelled RNA probes for the corresponding genes were generated by PCR, using genomic DNA as template isolated from *S. plymuthica* AS9 with the NucleoSpin® Tissue kit (Macherey-Nagel). Each reaction contained 0.2 μg DNA, 3 μL reaction buffer, 1 μL *Taq* polymerase (both from Thermo Scientific), 1 μL each of dATP, dCTP and dGTP, 6.5 μL dTTP (from 10 mM stock solutions), 3.5 μL of 1 mM DIG-11_dUTP (Roche, Mannheim, Germany), 2 μL of each primer (Table S4) and 24 μL of ddH_2O.

RNA isolation and Northern blot analysis

Total RNA was isolated using the hot phenol-SDS method [45] from *S. plymuthica* AS9 cultures (OD 0.005) grown in the presence or absence of (340.5 μg mL^{-1}) schleiferon A or B, as described above. Control experiments were treated with DMSO. RNA integrity was confirmed by agarose gel electrophoresis and quantified by spectrophotometry (Smart Spec 3000, BioRad), using 20 μg of the total RNA of each sample on a 1% gel. RNA was separated under denaturing conditions (72.5 mL running buffer (20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA × $2H_2O$, pH 7.0) and 2.5 mL formaldehyde). The RNA was transferred onto a positively charged nylon membrane (Roche, Mannheim, Germany) by capillary blotting using 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 16 h. Following the transfer, the RNA was cross-linked to the membrane by UV radiation (0.120 J/cm^2) for 1 min (Techne, Thermo-DUX, Wertheim, Germany). 16S rRNA bands on the membrane indicated the efficiency of the blotting and served as a control for the quantity of RNA in each lane. DIG-labelled probes were used to detect specific RNA molecules by hybridization [54].

Preparation of inverted membrane vesicles

E. coli TKR2000 was transformed with plasmid pNKN or pNKQ encoding wild-type LuxN or LuxQ, respectively. For overproduction of CqsS, *E. coli* Rosetta (DE3) pLysS was transformed with pKK-CqsS-6His encoding wild-type CqsS. Inside-out membrane vesicles were prepared as described previously [66].

Heterologous production of LuxP and LuxU

E. coli MDAI-2 was transformed with the plasmid pGEX_LuxP and purified as described elsewhere [42]. LuxU was overproduced using *E. coli* JM109 transformed with pQE30LuxU-6His, and purified as described by Timmen et al. [66]. All proteins were stored at −80 °C.

Phosphorylation assay

Phosphorylation reactions were performed in phosphorylation buffer (50 mM Tris/HCl pH 8.0, 10% (v/v) glycerol, 500 mM KCl, 2 mM DTT) at room temperature (69). The hybrid histidine kinases LuxQ, CqsS and LuxN were used as full-length membrane integrated proteins in inverted membrane vesicles at final concentrations of 5.5 mg mL^{-1} for LuxQ and CqsS and 2 mg mL^{-1} for LuxN. The reaction mixture contained 0.36 mg mL^{-1} LuxU and 0.48 mg mL^{-1} LuxP, unless otherwise indicated. To incorporate LuxP into LuxQ-containing membrane vesicles, three cycles of freezing and thawing were performed. Unless otherwise indicated, schleiferon A or B was

added at a final concentration of 340.5 μg mL^{-1} in DMSO (the corresponding volume of DMSO alone was used in the controls). The phosphorylation reaction was started by adding radiolabeled Mg^{2+}-ATP, typically 100 μM [γ-^{32}P]ATP (0.94 Ci $mmol^{-1}$; PerkinElmer, Rodgau-Jügesheim, Germany) and 110 μM $MgCl_2$, and stopped at various time points by the addition of SDS loading buffer, followed by fractionation of the reaction on SDS-polyacrylamide gels. Gels were dried at 80 °C on filter paper, exposed to a phosphoscreen for at least 24 h and scanned using a Typhoon Trio™ variable mode imager (GE Healthcare, München, Germany).

Bioluminescence assay

To compare the bioluminescence yields of wild-type *V. harveyi* ATCC BAA-1116 (BB120) (recently reclassified as *Vibrio campbellii* ATCC BAA-1116 [36]) with those of a *luxO*-deletion mutant (Δ*luxO*), cells from a culture grown overnight in LM medium (20 g L^{-1} NaCl, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract) were diluted to an OD_{600} of 0.05 in AB medium [20] and cultivated aerobically at 30 °C. Schleiferon A or B (at 340.5 μg mL^{-1}) was added to the exponential growth phase at the indicated time point. As a control, the corresponding volume of DMSO was added to the control culture. Cultivation as well as measurement of OD_{600} and luminescence (every 20 min) was performed in microtiter plates using a Tecan Infinite® F500 system (Tecan, Crailsheim). Data are reported as relative light units (RLU) in counts per second per milliliter per OD_{600}.

Statistical analysis

Comparisons between means were carried out according to the Student's t-test. Differences were considered significant at a p value of <0.05. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed using the web-based software tool MetaboAnalyst 3.0 [78].

Results

Volatile profiles of skin bacteria

The emission of VOCs into headspaces was comprehensively investigated for the two dominant families of the skin microbiota, Staphylococcaceae and Corynebacteriaceae. When grown in BHI broth, whose composition resembles that of human tissue, members of these families produced a variety of volatile compounds. The VOC profiles of *Corynebacterium* consisted of 11 different compounds (Table S1). Of these, 2-nonanone (#9), 2-phenylethanol (#12), 2-undecanone (#20), 8-pentadecanone (#46) and 2-pentadecanone (#47) were produced by all *Corynebacterium* isolates, and very large amounts of 2-phenylethanol were released by *Corynebacterium striatum* isolates (Fig. S1 (I–VII)). *Staphylococcus* strains emitted more than 50 compounds in all, among which ketones were dominant (Table S1). After a multivariate analysis, principal component analysis confirmed that Staphylococcaceae and Corynebacteriaceae could be partitioned into two distinct groups on the basis of their specific VOC profiles (Fig. 1A). Hierarchical clustering analysis also distinguished two clearly separated clusters corresponding to the two bacterial families. Moreover, each cluster was further divided into several subclusters according to the respective VOC profile of the bacterial isolate (Fig. 1B). Based on their VOC profiles, individual *C. striatum* and *Corynebacterium jeikeium* isolates, respectively, were more closely related to each other than to the other *Corynebacterium* species, while in the Staphylococcaceae family, representatives of the same bacterial species (e.g., *S. epidermidis*, *S. sciuri* or *S. schleiferi* isolates) did not always cluster together, and *S. sciuri* isolates together with *Staphylococcus intermedius* were distinctly separated from the other *Staphylococcus* species. Among the *Staphylococcus* strains, the VOC profiles of *S. schleiferi* isolates were particularly intriguing. Both strains produced more than 30 VOCs, among which were 2- and 3-methylbutanoic acids (#4 and #5), 2-phenylethylamine (#10), farnesol (#49) and several ketones (Table S2). The ketones #31, #35, #43 and #44 appeared in relatively high quantities and were only emitted by *S. schleiferi* isolates (Table S1). The structures of compounds #31 and #35 were elucidated as reported elsewhere (Schulz et al., in preparation). The two substances were identified as 3-(2-phenylethylamino)butan-2-one and (*E*)-3-(2-phenylethylimino)butan-2-one, and were named schleiferons A and B, respectively (Fig. 2A). Neither has previously been reported from natural sources. Schleiferons A and B were emitted in different amounts by the three *S. schleiferi* isolates DSMZ 4807, V431 and H34 (Figs. S2A and S2B, respectively), with DSMZ 4807 releasing approximately 30- to 100-fold higher levels of both compounds than V431 and H34, respectively, although all three strains had similar growth curves. During bacterial growth, schleiferons A and B first appeared in the stationary phase after 48 h incubation, and reached their maximum levels by 96 h. At 48 h, schleiferons A and B represented 34% and 2.4%, respectively, of the total VOC spectrum (Fig. 2B), which increased to 70% and 10% by 72 h, and 73% and 14% at 96 h.

Effects of S. schleiferi *volatiles on various microorganisms*

The effects of the bouquet of volatiles produced by a 24 h-old culture of *S. schleiferi* were evaluated on various Gram-positive and Gram-negative bacteria in co-culture experiments in which 96-well microtiter plates were inoculated with *S. schleiferi* and the test bacterium at the same time. After co-cultivation, the growth rates of the test species incubated with and without *S. schleiferi* did not differ significantly (Fig. S3A and B). Conversely, volatiles emitted by the tested species had no effect on the growth of *S. schleiferi* (Fig. S3C). Nevertheless, since schleiferons A and B were part of the volatile cocktail produced by *S. schleiferi* and were emitted in large quantities in the late stationary phase (Fig. 2B), co-culture experiments (in bipartite Petri dishes) were performed by inoculating a 96 h-old culture of *S. schleiferi* with *S. epidermidis* RP62A, *S. haemolyticus* CCM 2729 (Gram-positives), *S. enterica* RV4 or *S. marcescens* V11649 (Gram-negatives). After 24 h co-cultivation, *S. schleiferi* volatiles inhibited the growth of the Gram-positive bacteria significantly, but did not affect that of the Gram-negative species (Fig. 3A).

Effects of synthetic schleiferons A and B on Gram-positive bacteria

As documented in Fig. 3A, co-cultivation of *S. schleiferi* with other *Staphylococcus* strains significantly inhibited the growth of the latter. We therefore determined whether schleiferons A and B, when added to a liquid culture, were toxic for *Staphylococcus* isolates or for Gram-positive bacteria in general by testing their effects at different concentrations and determining their minimum inhibitory concentrations (MICs). Growth of all tested Gram-positive bacteria was indeed significantly inhibited by schleiferon A or B in a concentration-dependent manner (Fig. 3B, Table 1). Moreover, schleiferon A was approximately 10 times more effective than schleiferon B and both compounds were more toxic to corynebacteria and *Micrococcus luteus* than to staphylococci. The respective MICs ranged from 35 to 70 μM for schleiferon A, while *Staphylococcus*, *Enterococcus* and *Bacillus* strains were 2- to 16-fold less susceptible (Table 1). Interestingly, schleiferon A was less active on *S. schleiferi* DSMZ 4807 and *S. schleiferi* H34 than on *S. schleiferi* V431 and most other *Staphylococcus* strains. Among the *Staphylococcus* strains tested, schleiferon A was most effective against *S.*

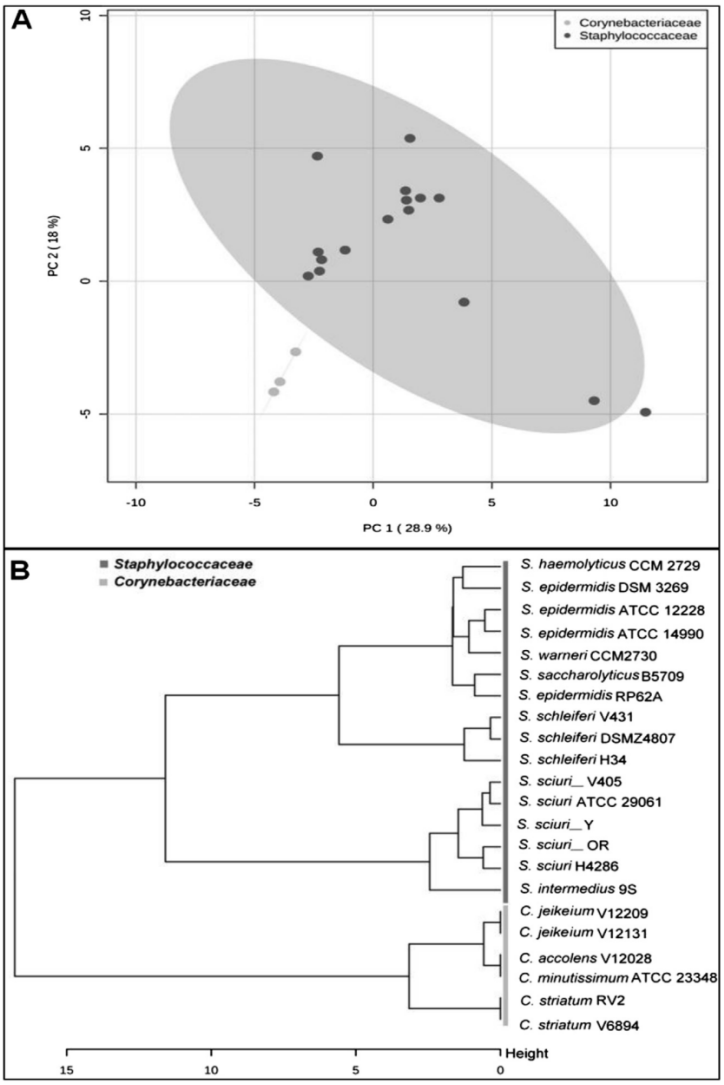

Fig. 1. Multivariate analysis of the volatile profiles of the Staphylococcaceae and Corynebacteriaceae families.
Multivariate analyses were performed using MetaboAnalist 3.0 [78], a comprehensive online tool for metabolomic data analysis. (A) Principal component analysis distinguished bacterial families on the basis of their VOC profiles. The shaded areas represent 95% of the confidence interval. (B) Dendrogram of bacterial species based on hierarchical clustering analysis of all VOCs emitted by the bacterial taxa investigated (original data in Table S1).

warneri, *S. epidermidis* and *S. haemolyticus*. In contrast, in a bipartite Petri-dish experiment, schleiferons A and B did not inhibit the growth of *S. epidermidis* and *S. haemolyticus* (Fig. S3D). Strikingly, when equivalent concentrations of schleiferon A or B were tested on Gram-negative bacteria (e.g., *S. enterica*), little or no growth inhibition was observed (Fig. 3B, Table 1).

S. schleiferi *volatiles repress quorum-sensing-dependent phenotypes of Gram-negative bacteria*

As shown in Fig. S4A, *S. schleiferi* VOCs had little effect on the growth of Gram-negative bacteria, while they significantly reduced the levels of prodigiosin produced by *S. marcescens* V11649. Prodigiosin is a red pigment (with a broad range of biological activities) produced by several bacteria, including *Serratia* strains, in which prodigiosin biosynthesis is known to be controlled by the quorum sensing system [61,75,76]. Therefore, the impact of VOCs was studied not only on prodigiosin synthesis in *S. marcescens* V11649 but also on bioluminescence in *V. harveyi* DSMZ 6904. When the former was co-cultivated with a 48 h-old culture of *S. schleiferi* for 24 h (Fig. S9), prodigiosin production was inhibited by approximately 40% (Fig. 4A and C) and repressed by more than 60% in a 96 h-old culture (Fig. 4B and D), indicating that the inhibitory effect depended on the concentration of VOCs present. Similarly, *V. harveyi* cultures showed an almost 60% reduction in bioluminescence when fumigated with *S. schleiferi* volatiles (Fig. 4E and F). It is important to note here that the bacterial cell density was not affected, and that exposure to volatiles from *S. warneri* had no impact on either prodigiosin synthesis or bioluminescence (Fig. 4).

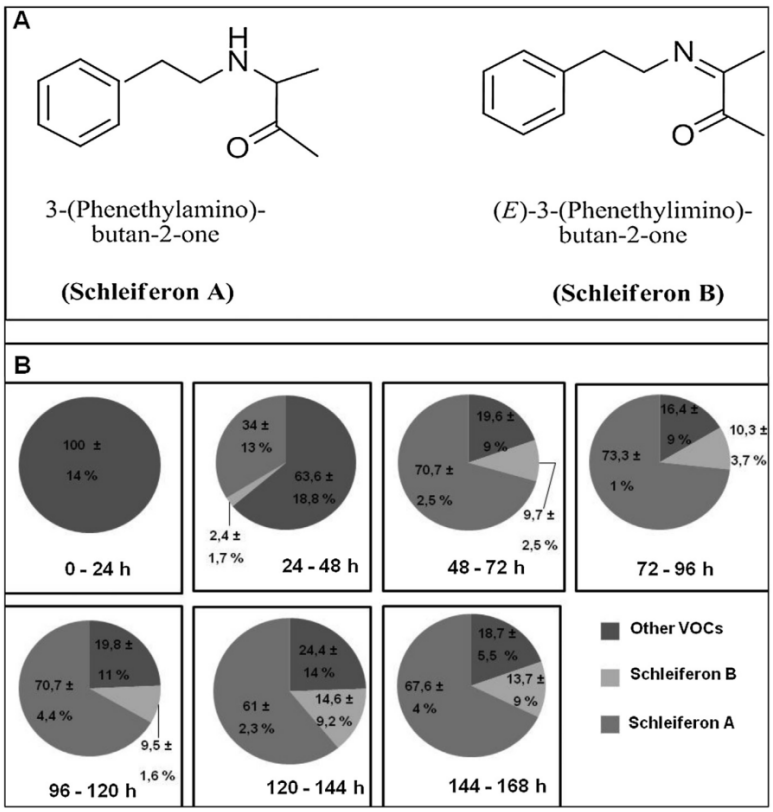

Fig. 2. Structure of schleiferons (A) and VOC spectrum of *Staphylococcus schleiferi* DSMZ 4807 (B).
VOCs were quantified and the relative contributions (%) of schleiferons A and B of the VOC spectrum of *S. schleiferi* cultures were determined at intervals of 24 h over the course of 1 week (based on data in Fig. S2) (100% (0–24 h) = 16.6 ng; 100% (24–48 h) = 34.2 ng; 100% (48–72 h) = 104.5 ng; 100% (72–96 h) = 130.3 ng; 100% (96–120 h) = 136.8 ng; 100% (120–144 h) = 109.3 ng; 100% (144–168 h) = 108.1 ng).

Table 1
Minimum inhibitory concentrations (MIC) of schleiferons A and B on different Gram-positive bacteria.

Bacteria		MIC (μM) of schleiferon	
Families	Species	A	B
Corynebacteriaceae	*Corynebacterium jeikeium* V12209	34.8	281
Corynebacteriaceae	*Corynebacterium striatum* RV2	34.8	281
Corynebacteriaceae	*Corynebacterium minutissimum* ATCC 23348	69.6	563
Staphylococcaceae	*Staphylococcus schleiferi* DSMZ 4807	278	1130
Staphylococcaceae	*Staphylococcus schleiferi* H34	278	2250
Staphylococcaceae	*Staphylococcus schleiferi* V431	139	2250
Staphylococcaceae	*Staphylococcus intermedius* 9S	139	563
Staphylococcaceae	*Staphylococcus saccharolyticus* B5709	139	1130
Staphylococcaceae	*Staphylococcus sciuri* V405	139	1130
Staphylococcaceae	*Staphylococcus warneri* CCM 2730	96.7	1130
Staphylococcaceae	*Staphylococcus epidermidis* RP62A	69.6	281
Staphylococcaceae	*Staphylococcus haemolyticus* CCM 2729	69.6	563
Micrococcaceae	*Micrococcus luteus* V515	34.7	141
Enterococcaceae	*Enterococcus faecium* ATCC 51559	278	2250
Enterococcaceae	*Enterococcus faecalis* ATCC 51299	557	3380
Bacillaceae	*Bacillus subtilis* B2g	278	1130
Enterobacteriaceae	*Salmonella enterica* RV4	4450	>10,000

Effects of synthetic schleiferons A and B on quorum-dependent phenotypes

To verify the hypothesis that schleiferons A and B emitted by *S. schleiferi* were responsible for the quorum-quenching effect of total VOCs on prodigiosin synthesis and bioluminescence emission by the test bacteria, cultures of *S. plymuthica* AS9, *S. marcescens* V11649 and *V. harveyi* DSMZ 6904 were incubated with different concentrations of pure (synthetic) schleiferons A and B. With increasing concentrations of the ketones, production of prodigiosin (Fig. S4B, 5A and B, S5A) and emission of bioluminescence (Fig. 5C, S5D), respectively, were eventually repressed to virtually undetectable levels. Inhibition of prodigiosin production by both *S. marcescens* (Fig. 5A) and *S. plymuthica* (Fig. 5B), as well as repres-

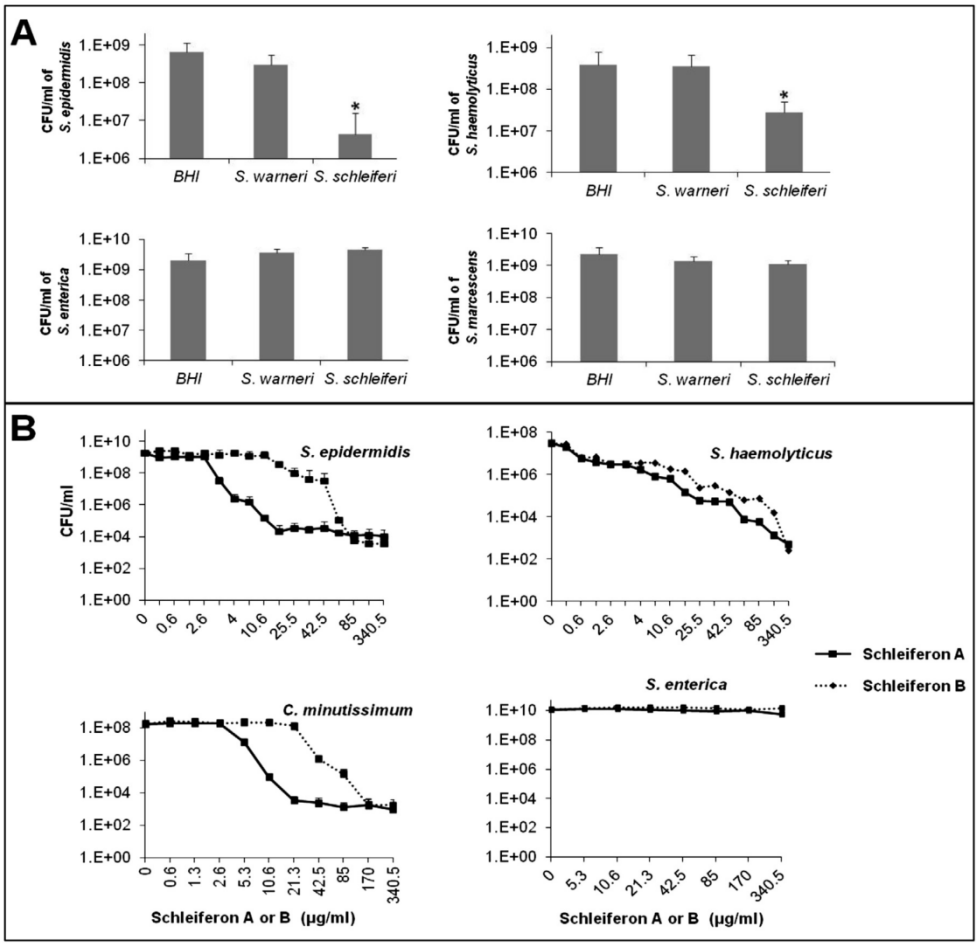

Fig. 3. Effects of *Staphylococcus schleiferi* and *S. warneri* volatiles (A) and synthetic schleiferons (B) on the growth of different Gram-positive and Gram-negative bacteria. (A) Gram-positive (*Staphylococcus epidermidis* RP62A, *Staphylococcus haemolyticus* CCM 2729) and Gram-negative (*Serratia marcescens* V11649, *Salmonella enterica* RV4) bacteria were co-cultivated with a 96-h old culture of *S. schleiferi* or *S. warneri* in bipartite Petri dishes for 24 h. Growth of the bacteria was monitored by determining viable cell numbers (CFU). Uninoculated growth medium (BHI) was used as a control. $^{*}p < 0.05$.
(B) *Staphylococcus epidermidis* RP62A, *Staphylococcus haemolyticus* CCM 2729, *Corynebacterium minutissimum* ATCC 23348 and *Salmonella enterica* RV4 were grown at 37 °C in BHI medium in the presence of the indicated final concentrations of schleiferon A or B. After 20 h, viable cell numbers (CFU) were determined. Data are the means of three independent experiments and bars indicate the mean standard deviation. When error bars are not visible, they were smaller than the symbols.

sion of bioluminescence in *V. harveyi* (Fig. 5C), was already obvious at a low concentration (5.3 µg mL^{-1}) of the test substance and increased gradually with an increasing concentration of added schleiferon A or B. At 42.5 µg mL^{-1}, both phenotypes were suppressed by approximately 50% and at the maximum concentration (340.5 µg mL^{-1}) almost no prodigiosin or bioluminescence was detected, while the cell density was only slightly affected by schleiferon A or B. Moreover, determination of the numbers of viable cells in *S. marcescens* (Fig. S5B) and *S. plymuthica* (Fig. S5C) cultures treated with schleiferon A or B (CFU mL^{-1}) revealed no significant differences compared to the control (bacteria treated with DMSO).

Schleiferons A and B act downstream of the quorum-sensing phosphorelay of V. harveyi

Induction of bioluminescence in *V. harveyi* depends on a complex quorum-sensing (QS) signaling cascade. At low cell density, in the absence of autoinducers, the three hybrid histidine kinases LuxN, LuxQ (in interplay with LuxP) and CqsS autophosphorylate the transfer of the phosphoryl group via a phosphorelay to the histidine phosphotransferase protein (HPr) LuxU, and subsequently to the response regulator LuxO (69). Phosphorylated LuxO activates transcription of five regulatory sRNAs which, together with the RNA chaperone Hfq, destabilize the transcript coding for the master regulator LuxR. When the concentration of LuxR in cells is low, induction of bioluminescence is impossible. At high cell density, in the presence of high concentrations of autoinducers, autophosphorylation is inhibited, LuxR is synthesized, and the bioluminescence phenotype is expressed [72]. To gain insight into the molecular mechanism of schleiferon A- and B-mediated inhibition of bioluminescence in *V. harveyi*, in vitro phosphorylation assays of the three hybrid histidine kinases were performed. LuxQ, CqsS and LuxN, respectively, were heterologously expressed in *E. coli*, and inverted membrane vesicles bearing the full-length proteins were

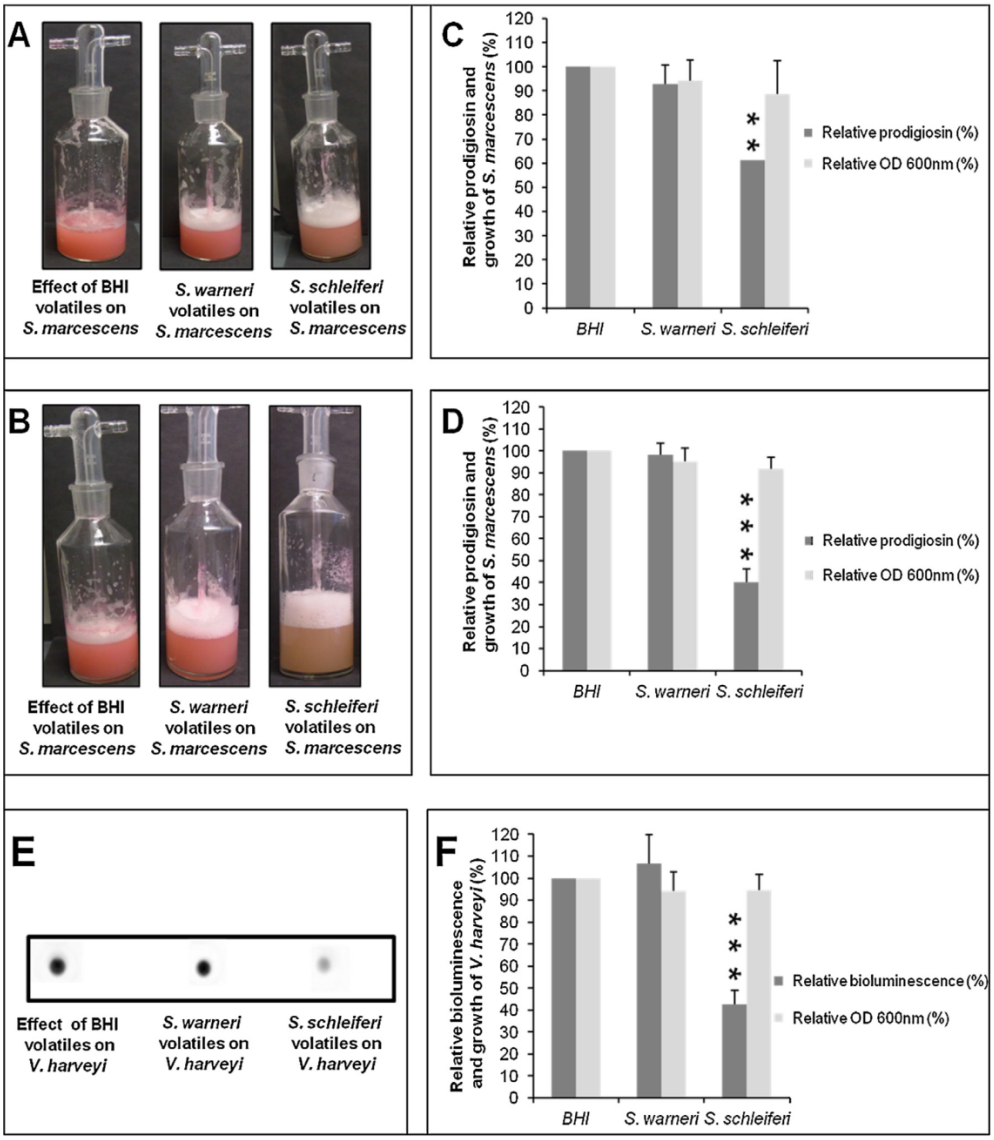

Fig. 4. Volatiles of *Staphylococcus schleiferi* inhibit prodigiosin production in *Serratia marcescens* and bioluminescence in *Vibrio harveyi*.
S. marcescens was cultivated in a dual culture system (Fig. S9) for 24 h with a 48-h (A) or a 96-h (B) culture of *S. schleiferi* or *S. warneri* (BHI: brain heart infusion medium). Cell density (OD_{600} of 100% = 4) and relative levels of prodigiosin in the culture medium (%) (OD_{534} of 100% = 1.4) for *S. marcescens* were measured after 24 h dual cultivation of *S. marcescens* with a 48 h (C) or 96 h (D) culture of *S. schleiferi*/*S. warneri*. (E) shows the light image of *V. harveyi* bioluminescence cultivated in a dual culture system (Fig. S9) for 24 h with a 96-h old culture of *S. schleiferi*/*S. warneri*, and (F) shows the relative growth (% cell density) (OD_{600} of 100% = 5) and bioluminescence emission (%) (100% = 7.9E + 05) for *V. harveyi*. The culture medium was used as a control. Data are the means of 3–5 independent experiments and bars indicate the mean standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$.

directly used for phosphorylation assays. The effect of schleiferons A and B (340.5 μg mL^{-1} final concentration) was investigated on the autophosphorylations catalyzed by the three kinases and on the LuxU phosphotransfer reaction. However, no significant increase was detected in phosphorylated LuxU compared to the controls, which were treated with DMSO alone (Fig. S6A–C).

Moreover, in an independent in vivo assay, it was found that bioluminescence of the *luxO* deletion mutant, which exhibits autoinducer-independent (i.e., constitutive) bioluminescence, was still inhibited by application of either schleiferon (Fig. S7). These results demonstrated that the decrease in bioluminescence caused by schleiferons A and B was not related to any perturbation of the QS phosphorelay by these compounds.

Synthetic schleiferons A and B stimulate the regulators of prodigiosin biosynthesis in S. plymuthica *AS9*

Prodigiosin biosynthesis has been well characterized in *Serratia* sp. strain ATCC 39006 [61,75,76]. Homologous genes involved in the biosynthesis of this red pigment are found in *S. plymuthica* AS9 [29]. The *pig* cluster (*pigA-O*) encodes the enzymes that catalyze the synthesis of the pigment from proline and octenal to prodigiosin,

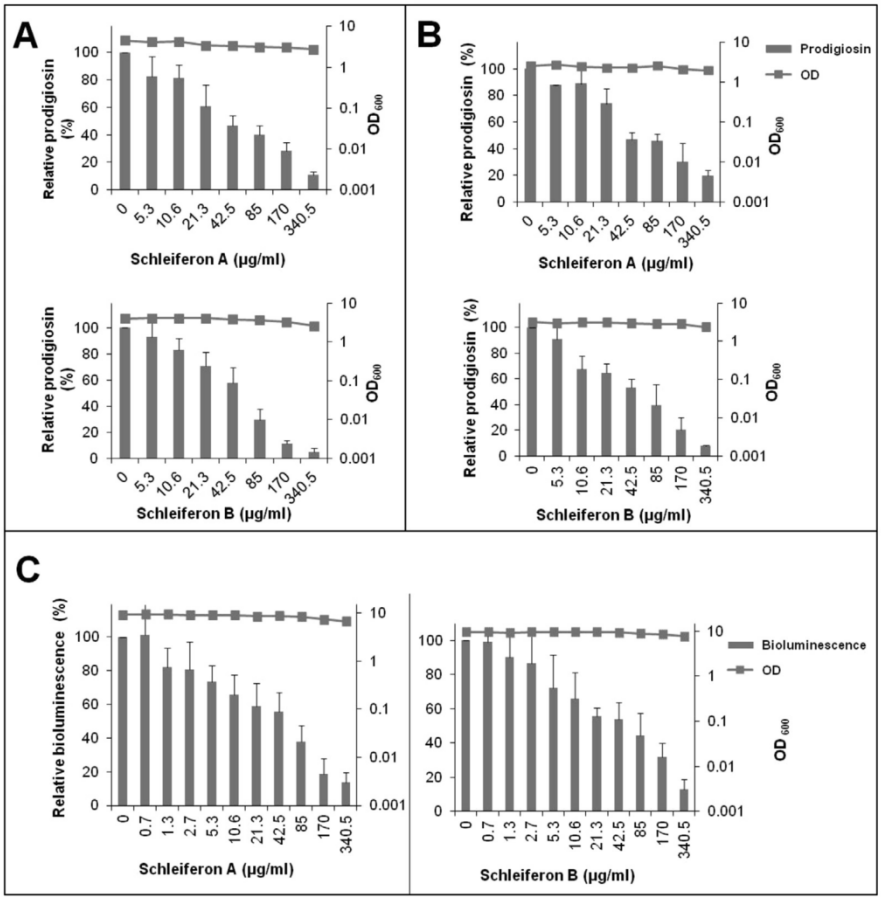

Fig. 5. Inhibition of prodigiosin production in *Serratia marcescens* and *S. plymuthica* (A and B) and bioluminescence emission by *Vibrio harveyi* (C) by synthetic schleiferons A and B.
Bacteria were incubated with increasing concentrations of schleiferon A or B (final concentrations are shown). Bacterial growth was monitored by measuring the cell density at OD_{600}, prodigiosin levels were determined from the OD_{534} value and relative prodigiosin production was calculated as the ratio between prodigiosin and cell density (100% = 2 for *S. marcescens* (A) and 0.98 for *S. plymuthica* AS9 (B)). Relative bioluminescence emission (%) (100% = 8.7E + 05) was calculated as the ratio between bioluminescence intensity and cell density (C). Controls: bacteria were incubated with DMSO and their prodigiosin or bioluminescence emission levels were set as 100%. Data are means of at least five independent experiments and bars indicate the mean standard deviation.

while *pigP*, *pigS* and *luxR* code for transcriptional regulators of the cluster and *luxS* encodes the synthase responsible for production of the quorum-sensing auto-inducer AI-2 [23,61]. It was hypothesized that schleiferons A and B might repress prodigiosin synthesis by acting on the transcription factors. To test this idea, Northern blot analysis was used (Fig. 6) to determine the levels of the *pigP*, *pigS* and *luxR* transcripts. As a result, increased *pigP*, *pigS* and *luxS* mRNA levels were observed after application of schleiferon B, while the *luxR* level was unaffected (Fig. 6A). Furthermore, the expression levels of the prodigiosin gene cluster of *S. plymuthica* AS9 were investigated. Since the biosynthetic genes are co-transcribed [61], the transcription level of one of the genes (*pigG*) was analyzed using Northern blotting and a decrease was found in the *pigG* transcript (Fig. 6B), which consequently indicated a decrease in transcription of the *pig* gene cluster.

Discussion

In this study, it was shown that skin bacteria were a rich source of natural volatile organic compounds (VOCs) and novel compounds were identified that selectively inhibited the growth of Gram-positive bacteria and influenced specific phenotypes (prodigiosin synthesis, bioluminescence) in Gram-negative bacteria. It was demonstrated that schleiferons A and B, volatiles emitted by *S. schleiferi*, had detectable effects on other bacterial species that colonize the skin. Similar results have been reported by Chernin et al. [11] for rhizobacterial volatiles acting on plants, or have been reviewed by Audrain et al. [3]. These results underlined the need to take volatile-based modes of interaction and communication into account when considering microbiome establishment and dynamics.

While 2-pentadecanone was emitted by all skin bacteria investigated in this study, some VOCs were found to be strain and/or species specific. In particular, fatty carboxylic acids, such as 3-methylbutanoic and 2-methylbutanoic acid, were produced by all *Staphylococcus* strains examined. This confirmed the finding of Ara et al. [1], who showed that *S. epidermidis* was able to metabolize the amino acid leucine present in sweat secretions to 3-methylbutanoic acid, which is also the main component of foot odor. In contrast to staphylococci, not all corynebacteria generated and emitted

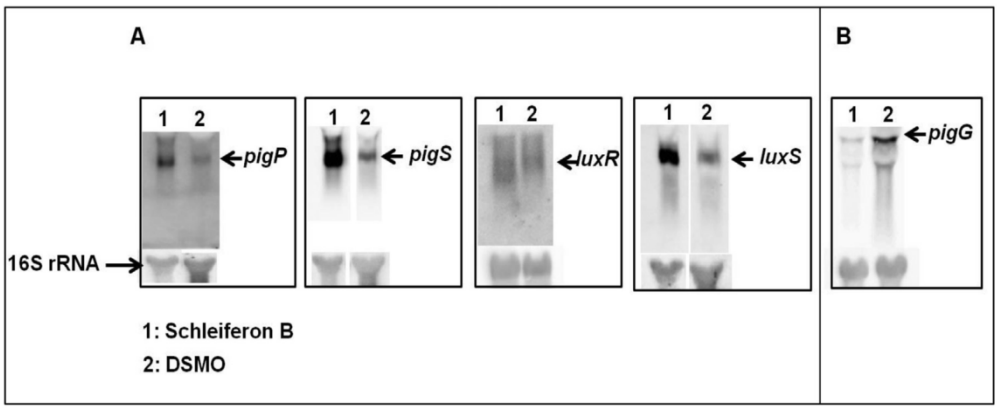

Fig. 6. Expression levels of the prodigiosin regulatory genes (A) and biosynthetic genes (B) in *Serratia plymuthica* AS9.
The bacterium was incubated at 20 °C with schleiferon A or B (340 μg mL^{-1} final concentration). As a control, the corresponding volume of DMSO was added to the cells. After 24 h, total RNA was extracted and Northern blot analysis was performed to quantify the expression of the genes (c) involved in the regulation (A) and biosynthesis (B) of prodigiosin. (A) *pigP*, *pigS*, *luxR*, and *luxS* correspond to the genes SerAS9, SerAS9_1501, SerAS9_0078 and SerAS9_0772, respectively. (B) *pigG* corresponds to the gene *SerAS9*_1747 in the *pig* biosynthesis cluster. The bands of 16S rRNA served as an indication of the blotting efficiency and demonstrated the loading of RNA in each lane.

these VOCs. This finding highlights the important role of staphylococci in foot odor formation. However, 2-phenylethanol was emitted by all *Corynebacterium* strains analyzed here and was produced in particularly large amounts by *C. striatum* isolates, while among staphylococci only *S. schleiferi* and *S. sciuri* isolates were able to produce this compound. Early studies had shown that 2-phenylethanol is in general a common bacterial volatile [58] and that it is present in the headspace of the skin microbiome [68]. In light of the results presented here, it is likely that 2-phenylethanol belongs to the cocktail of volatiles generated by bacterial residents of the skin, and is mainly produced by corynebacteria. This volatile has a characteristic rose-like odor and has been shown to act as a mosquito repellent [69]. Moreover, this compound has been reported to possess antimicrobial, antiseptic and disinfectant properties [19,39], which has prompted speculation that it may be emitted by corynebacteria during interspecies competition for niches on the skin in order to eliminate other competitors and/or inhibit the growth of pathogens on the skin.

Another interesting observation was that the VOC profiles of the two dominant families of skin bacteria could be easily distinguished. This difference can probably be explained by metabolic differences between the two genera, even though they share the same environment and the same growth conditions. Moreover, this is in good agreement with previous studies, which showed that the spectrum of volatiles emitted by bacterial communities depends not only on growth conditions and growth medium but also on the metabolic activities of their constituent species [9,10,65,73]. Furthermore, the work of Kwaszewska et al. [31] demonstrated that corynebacterial and staphylococcal communities co-habiting on human skin do not employ the same sets of enzymatic activities to metabolize substrates such as carbohydrates, lipids and proteins, and that *Corynebacterium* species express less proteinase, phospholipase and saccharolytic activity. These observations could also explain why the number of VOCs emitted by the *Staphylococcus* strains studied here was fivefold higher than that of the corynebacterial strains, allowing members of the two genera to be clearly differentiated by PCA on the basis of their VOC spectra alone (Fig. 1).

In the course of the analysis of the VOCs of these species, two new volatiles were identified – schleiferons A and B – which have never previously been associated with any biological source. In the sample of taxa, these substances were only produced by *S. schleiferi* isolates. On the basis of other work, Schulz et al. (unpublished) have postulated a biosynthetic route for these compounds via acetoin and 2-phenylethylamine, both of which are produced by *S. schleiferi*. Acetoin is an essential physiological metabolite produced by many bacteria and serves as a precursor in the biosynthesis of branched-chain amino acids. Although it is primarily formed by decarboxylation of alpha-acetolactate, it can also be secreted as a by-product of pyruvate oxidation or decarboxylation reactions [reviewed in Ref. [79]]. On the other hand, instances of bacterial production of 2-phenylethylamine are far less prominent in the literature, and the capacity to synthesize it is not widely distributed even among staphylococci [7,32,63]. Nevertheless, some enterococci, as well as lactic acid bacteria isolated from food products, have been shown to synthesize this compound via decarboxylation of L-phenylalanine by tyrosine decarboxylase [4,33,38,47]. Thus, we speculate that 2-phenylethylamine is synthesized by *S. schleiferi* using the same mechanism or via an as yet undescribed specific L-phenylalanine decarboxylase. Acetoin appears early in the VOC profile of *S. schleiferi* DSMZ 4807, after 24 h growth. In bacteria, this compound is usually produced during exponential growth in order to prevent over-acidification of the cytoplasm and the surrounding medium before the major carbon source has been used up [79]. However, 2-phenylethylamine was only detected after 48 h, which could explain why schleiferons A and B only attained their maximum levels in the late stationary phase of bacterial growth. Since schleiferon A already comprised up to 30% of volatiles at 48 h (Fig. 2B), sufficient 2-phenylethylamine must have been available prior to that time.

Microbe-microbe interactions are known to be mediated via secondary metabolites [56]. To gain insight into the biological role(s) of schleiferons A and B, *S. schleiferi* DSMZ 4807 was co-cultivated with bacteria that are naturally found on the skin, and with other microorganisms from different microbiota. The results revealed that *S. schleiferi* volatiles selectively inhibited the growth of Gram-positive bacteria. The growth inhibition was only significant when the bacteria were co-cultivated with *S. schleiferi* cells that were in the late stationary phase, the period in which the concentrations of schleiferons A and B reached their maxima. This suggested that these compounds might contribute to the reduced growth rate of Gram-positive species. This notion was further supported by the finding that *S. warneri*, which does not synthesize either schleiferon, did not affect any of the bacteria tested. Moreover, when chemically synthesized schleiferons A

and B were tested separately, they both specifically inhibited the growth of Gram-positive bacteria in a concentration-dependent manner, with schleiferon A being significantly more active than schleiferon B.

During competition for nutrients or territory, skin bacteria release different antimicrobial compounds to prevent adherence of pathogens and/or competitors [8,13]. Schleiferons were significantly more active (3- to 16-fold) against *Corynebacterium* strains and *M. luteus* (another skin bacterium) and less deleterious to *Bacillus subtilis* (rhizobacterium) and *Enterococcus* strains (gut bacteria). Moreover, they were also active against other skin *Staphylococcus* strains, although the latter were considerably less affected than corynebacteria or *M. luteus*. Therefore, the production of schleiferons can be an advantage for staphylococci during bacterial interactions and might help them retain their balance in the skin flora. In addition, among these skin bacteria, the prominent schleiferon producer *S. schleiferi* DSMZ 4807 was 2- to 8-fold more resistant to these VOCs and its growth was noticeably affected at very high concentrations, which was only be reached in the late stationary phase. Altogether, these results highlighted the role(s) that schleiferons may have in skin bacterial interactions, suggesting that mVOCs might contribute to maintain species diversity and to shape the evolution of the community composition or structure the microbiome. However, the mode of action by which these compounds inhibit the growth of Gram-positive bacteria is not yet known and remains to be investigated in the future.

In contrast, the growth of Gram-negative bacteria was unaffected by either schleiferon A or B, although both compounds inhibited quorum-dependent phenotypes, specifically prodigiosin production in *Serratia* strains and bioluminescence by *V. harveyi*. Incubation of cell extracts with schleiferon A or B did not alter their prodigiosin content (results not shown), implying that the two agents disrupted its synthesis at the transcriptional or post-transcriptional level. Homologues of the prodigiosin biosynthesis genes (*pigA-N*) of *Serratia* sp. ATCC 39006 are present in *S. marcescens* and *S. plymuthica* [29,35,76] and the genes in these clusters are co-transcribed and directed by a promoter upstream of *pigA* [61]. The Northern blot analysis revealed that the transcription level of *pigG* (representing the co-transcribed *pig* cluster) was significantly inhibited in the presence of schleiferon B (Fig. 6B), which ultimately led to the reduction of prodigiosin synthesis and accumulation. A complex hierarchical network of regulatory proteins control the biosynthesis of prodigiosin [17,23,75] and, since the production of this pigment is regulated by both the quorum-dependent and -independent mechanisms, the transcription levels of both master transcriptional regulator genes *pigP* and *luxR* were analyzed. Fineran et al. [17] previously identified the PigP protein as a master transcriptional regulator of secondary metabolism in some Enterobacteriaceae. They found that this protein could control prodigiosin production in *Serratia* sp. ATCC 39006 either by directly regulating the expression of the *pig* biosynthesis cluster or indirectly via transcriptional control of genes for six other regulators (*pigQ*, *pigR*, *rap*, which overlap the quorum-sensing circuit, and *pigV*, *pigS* or *pigX*). When schleiferon was added to a *S. plymuthica* AS9 culture, the transcriptional level of *pigP* was significantly increased in comparison to the control, suggesting control via this *pig* regulator. Moreover, when the transcription levels of *pigS* were analyzed, a significant increase was also found in its transcription level. *pigS* encodes for an ArsR family regulator that is able to repress the expression of other proteins (BlhA; OrfY; PmpA, B and C) involved in prodigiosin biosynthesis [23]. Therefore, the transcription level of *pigS* correlated with the activation of the expression of the master regulator PigP, and both up-regulations might favor a reduction of prodigiosin in *S. plymuthica* AS9. Similarly to *pigP*, the expression of *luxS* (encoding for the auto-inducer 2 synthase) was also up-regulated. In contrast to PigP, SmaR in *Serratia* sp. ATCC 39006 (*LuxR* homolog in *S. plymuthica* AS9) is a quorum-sensing master transcriptional repressor [17,23,61]. LuxR represses expression of the *pig* biosynthesis gene cluster directly or indirectly via the repression of other transcriptional regulators, such as PigR, PigQ or Rap [17]. When the transcription levels of *luxR* were analyzed in *S. plymuthica* AS9 after application of schleiferon, it was found surprisingly that they were almost unaffected. Altogether, these results support the notion that in Gram-negative bacteria exposed to schleiferon A or B, the expression of the master regulator PigP was stimulated and led to the inhibition of prodigiosin production. Nevertheless, the transcriptional regulators that overlap the quorum-dependent circuit that are under the control of PigP still have to be investigated.

The discussion has so far concerned molecular circuits in the context of how schleiferons A and B induced the repression of prodigiosin biosynthesis. Nevertheless, it can also be postulated whether or not schleiferon could act on other targets of quorum sensing (e.g., as an auto-inducer antagonist) by interfering with auto-inducer receptors to influence prodigiosin or bioluminescence production through stimulation of autophosphorylation [12,72]. Surprisingly, no increase in phosphorylation of the quorum sensing receptors was found after schleiferon A or B addition, suggesting a target(s) downstream of the hybrid histidine kinase. These results were corroborated by the schleiferon-mediated inhibition of bioluminescence in a *luxO* deletion mutant, which constitutively produces bioluminescence independently of auto-inducers and the QS receptors.

In summary, it has been shown that a large number of VOCs were present in the headspace of skin bacteria, including bio-organic compounds that have never been recognized before in natural settings. Schleiferons A and B selectively inhibited bacterial growth or affected bacterial metabolism and gene expression. Considering the problem of continued emerging antibiotic-resistant pathogens, it would be very interesting to test these new natural compounds on pathogenic strains. As these mVOCs interfere in bacterial communication by affecting gene expression, their impact on the expression of virulence factor genes needs to be determined in order to evaluate their efficacy in anti-virulence therapy. The local microbiota plays an important role in buttressing the skin's role as a barrier against colonization by different microorganisms and invasion by pathogens by engaging in competitive interactions that control access to nutrients and/or space. Future experiments will need to elucidate the relevance of these microbial volatiles to such interactions on the skin, and will contribute to a better understanding of the implications of the microbiota in health and disease conditions.

Contributions

MK and MCL performed the PCA and the hierarchical clustering analysis (Fig. 1), SRR and SS elucidated the structure (Fig. 2A) and helped to analyze the VOCs of the headspaces (Tables S1 and S2), whereas NL and KJ performed the phosphorylation experiments and the in vitro bioluminescence assay (Figs. S6 and S7, respectively). All other experiments were performed by MCL. MCL and BP planned the experiments and wrote the manuscript. All authors interpreted the results and approved the submitted version of the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2016.08.008.

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Supporting information

Table S1: VOC profiles of skin bacteria analyzed by GC/MS.

Compounds			*Staphylococcaceae*																*Corynebacteriaceae*					
No.	**Names**	**RI**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**
1	Acetoin	708	x		x							x				x	x	x						
2	1,2-Propanediol	772										x		x			x							
3	2,3-Butanediol	782															x	x						
4	3-Methylbutanoic acid	812	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				
5	2-Methylbutanoic acid	819	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				
6	NI	927									x	x	x	x		x	x	x						
7	4-Methylhexanoic acid	1011										x												
8	NI	1046	x																					
9	2-Nonanone	1064																	x	x	x	x	x	x
10	2-Phenylethylamine	1084	x	x																				
11	NI	1088	x	x																				
12	2-Phenylethanol	1095	x		x	x	x	x	x	x									x	x	x	x	x	x
14	NI	1128	x	x				x				x												
15	7-Methyl-2-nonanone	1134	x								x	x	x	x	x		x	x						
16	NI	1196										x												
17	NI	1223					x	x	x	x	x							x						
18	2-Phenylethyl acetate	1234																	x	x				
19	NI	1258													x									
20	2-Undecanone	1260	x	x	x						x	x	x	x		x	x	x	x	x	x	x	x	x
21	NI	1319													x									
22	10-Methyl-2-undecanone	1322	x	x	x			x			x	x	x	x			x	x						

Table S1: VOC profiles of skin bacteria analyzed by GC/MS (cont.).

Compounds		*Staphylococcaceae*																*Corynebacteriaceae*					
No.	**Names**	**RI**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**
23	NI	1327													x									
24	9-Methyl-2-undecanone	1329	x	x	x			x			x	x	x	x		x	x	x						
25	NI	1355													x		x							
26	Methyl ester	1357															x							
27	NI	1416													x	x								
28	NI	1419	x	x																				
29	6-Tridodecanone	1434																	x	x				
30	NI	1452													x	x								
31	(E)-3-(Phenylethylimino)butan-2-one	1456	x	x	x																			
34	NI	1469	x	x	x																			
35	3-(Phenylethylamino)butan-2-one	1486	x	x	x																			
36	NI	1514													x									
37	11-Methyl-2-tridecanone	1518	x	x	x	x	x	x	x		x	x	x	x		x	x	x						
38	NI	1525													x									
39	10-Methyl-2-tridecanone	1527	x	x	x	x	x	x	x		x		x	x	x	x	x	x						
40	NI	1540	x	x																				
41	NI	1548									x						x							
42	Methyl ester	1555									x						x							
43	3-(Phenylethylamino)pentan-2-one	1556	x	x																				
44	4-(Phenylethylamino)pentan-3-one	1574	x	x																				
45	NI	1618	x	x																				
46	8-Pentadecanone	1630																	x	x	x	x	x	x

Table S1: VOC profiles of skin bacteria analyzed by GC/MS (cont.).

Compounds			*Staphylococcaceae*																*Corynebacteriaceae*					
No.	**Names**	**RI**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**
47	2-Pentadecanone	1653	x	x	x	x			x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
49	Farnesol	1674	x	x	x							x	x	x	x	x		x						
50	NI	1707				x									x									
51	14-Methyl-2-pentadecanone	1713	x	x	x						x	x	x	x		x	x	x						
52	NI	1716				x		x	x	x					x									
53	13-Methyl-2-pentadecanone	1722	x	x	x						x	x	x	x		x	x	x						
54	NI	1752	x	x	x																			
55	NI	1773	x																					
56	NI	1841	x									x		x										
57	Heptadecen-2-one*	1857																			x	x	x	x
58	NI	1861				x	x	x	x	x					x									
59	2-Heptadecanone	1868																			x	x	x	x
60	NI	1869	x	x	x						x	x	x	x	x	x	x	x						
61	16-Methyl-2-heptadecanone	1915	x	x							x	x	x	x										
62	NI	1916				x	x	x	x	x			x	x			x	x						
63	15-Methyl-2-heptadecanone	1917	x			x		x	x	x	x	x	x	x			x	x						
64	NI	1965	x	x																				
65	NI	2029	x	x																				
66	2-Tridodecanone	1460																			x	x		

NI: not identified. **A**= *Staphylococcus schleiferi* DSMZ 4807; **B**= *S. schleiferi* V431; **C**= *S. schleiferi* H34; **D**= *S.sciuri* ATCC 29061; **E** = *S. sciuri* H4286; **F**= *S. sciuri* OR; **G**= *S. sciuri* V405; **H**= *S. sciuri* Y; **I**= *S. epidermidis* RP62A; **J**= *S. epidermidis* DSM 3269; **K**= *S. epidermidis* ATCC 14990; **L**= *S. epidermidis* ATCC 12228; **M**= *S. intermedius* 9S; **N**= *S. haemolyticus* CCM 2729; **O**= *S. saccharolyticus* B5709; **P**= *S. warneri* CCM 2730; **Q**= *Corynebacterium striatum*

ATCC 12228; **M**= *S. intermedius* 9S; **N**= *S. haemolyticus* CCM 2729; **O**= *S. saccharolyticus* B5709; **P**= *S. warneri* CCM 2730; **Q**= *Corynebacterium striatum* V6894; **R**= *C. striatum* RV2; **S**= *C. minutissimum* ATCC 23348; **T**= *C. accolens* V12028; **U**= *C. jeikeium* V12131; **V**= *C. jeikeium* V12209. *Position of double bond unknown. *S. schleiferi* isolates were grown on BHI medium for 120 h at 30 °C. Volatiles in the headspace of *S. schleiferi* cultures were analyzed at 24 h intervals by GC/MS. Compounds were numbered as shown in the chromatograms (Fig. S1 (IIX-XXIV)) and were identified by comparing their retention index (RI) and mass spectra with the NIST 107 mass spectral library (version 1998), NIST Chemistry WebBook [44], and with authentic standards.

Table S2: Volatiles of three *Staphylococcus schleiferi* isolates.

Compound			*S. schleiferi* isolates		
Number	**Compound name**	**RI**	**DSMZ 4807**	**V431**	**H34**
1	Acetoin	708	*		*
4	3-Methylbutanoic acid	812	*	*	*
5	2-Methylbutanoic acid	819	*	*	*
8	NI	1046	*		
10	2-Phenylethylamine	1084	*	*	
11	NI	1088	*	*	
12	2-Phenylethanol	1095	*		*
14	NI	1128	*	*	
15	7-Methyl-2-nonanone	1134	*		
20	2-Undecanone	1260	*	*	*
22	10-Methyl-2-undecanone	1322	*	*	*
24	9-Methyl-2-undecanone	1329	*	*	*
28	NI	1419	*	*	
31	(E)-3-(Phenylethylimino)butan-2-one	1456	*	*	*
34	NI	1469	*	*	*
35	3-(Phenylethylamino)butan-2-one	1486	*	*	*
37	11-Methyl-2-tridecanone	1518	*	*	*
39	10-Methyl-2-tridecanone	1527	*	*	*
40	NI	1540	*	*	
43	3-(Phenylethylamino)pentan-2-one	1556	*	*	
44	4-(Phenylethylamino)pentan-3-one	1574	*	*	
45	NI	1618	*	*	
47	2-Pentadecanone	1653	*	*	*
49	Farnesol	1674	*	*	*
51	14-Methyl-2-pentadecanone	1713	*	*	*
53	13-Methyl-2-pentadecanone	1722	*	*	*
54	NI	1752	*	*	*
55	NI	1773	*		
56	NI	1841	*		
60	NI	1869	*	*	*
61	16-Methyl-2-heptadecanone	1915	*	*	
63	15-Methyl-2-heptadecanone	1947	*		
64	NI	1965	*	*	
65	NI	2029	*	*	

S. schleiferi isolates were grown in BHI medium for 120 h at 30 °C. Volatiles in the headspace of *S. schleiferi* cultures were analyzed at 24 h intervals by GC/MS. Compounds

were numbered as shown in the chromatograms (Fig. S1 (IIX-XXIV)) and were identified by comparing their retention index (RI) and mass spectra with the NIST 107 mass spectral library (version 1998), NIST Chemistry WebBook [44], and with authentic standards. NI: not identified.

Table S3: Bacterial strains used in this study (cont.).

Genus	Species	Isolate	Origin#	Isolation site
Corynebacterium	*jeikeium*	V12209	University of Rostock	Clinical isolate
Corynebacterium	*jeikeium*	V12131	University of Rostock	Clinical isolate
Corynebacterium	*striatum*	RV2	University of Rostock	Clinical isolate
Corynebacterium	*striatum*	V6893	University of Rostock	Clinical isolate
Corynebacterium	*accolens*	V12028	University of Rostock	Clinical isolate
Corynebacterium	*minutissimum*	ATCC 23348	ATCC	Trunk of adult female
Staphylococcus	*saccharolyticus*	B5709	University of Rostock	Clinical isolate
Staphylococcus	*schleiferi*	DSMZ 4807	DSMZ	Catheter
Staphylococcus	*schleiferi*	H34	University of Rostock	Clinical isolate
Staphylococcus	*schleiferi*	V431	University of Rostock	Clinical isolate
Staphylococcus	*intermedius*	9S	University of Rostock	Clinical isolate
Staphylococcus	*sciuri*	V405	University of Rostock	Clinical isolate
Staphylococcus	*sciuri*	H4286	University of Rostock	Clinical isolate
Staphylococcus	*sciuri*	OR	University of Rostock	Clinical isolate
Staphylococcus	*sciuri*	H4286	University of Rostock	Clinical isolate
Staphylococcus	*sciuri*	ATCC 29061	ATCC	Southern flying squirrel skin
Staphylococcus	*warneri*	CCM 2730	CCM	Human skin
Staphylococcus	*epidermidis*	RP62A	ATCC	Catheter
Staphylococcus	*epidermidis*	DSM 3269	DSMZ	Catheter
Staphylococcus	*epidermidis*	ATCC 14990	ATCC	Nose
Staphylococcus	*epidermidis*	ATCC 12228	ATCC	–

Table S3: Bacterial strains used in this study (cont.).

Staphylococcus	*haemolyticus*	CCM 2729	CCM	Human skin
Micrococcus	*luteus*	V515	University of Rostock	Clinical isolate
Enterococcus	*faecium*	ATCC 51559	ATCC	Clinical isolate
Enterococcus	*faecalis*	ATCC 51299	ATCC	Peritoneal fluid
Pseudomonas	*fluorescens*	V12141	University of Rostock	Clinical isolate
Serratia	*marcescens*	V11649	University of Rostock	Clinical isolate
Salmonella	*enterica*	RV4	University of Rostock	Clinical isolate
Escherichia	*coli*	DH5α	Laboratory strain	–
Vibrio	*harveyi*	DSMZ 6904	DSMZ	Seawater
Vibrio	*harveyi* BB120	ATCC BAA-1116*	ATCC	Marine (ocean)
Vibrio	*harveyi* BB120*ΔluxO*	ATCC BAA-1116*		[46]
Serratia	*plymuthica*	AS9	Swedish University of Agricultural Sciences	Field sample, rapeseed roots
Bacillus	*subtilis*	B2g	SCAM, University of Rostock	Rhizosphere of oilseed rape

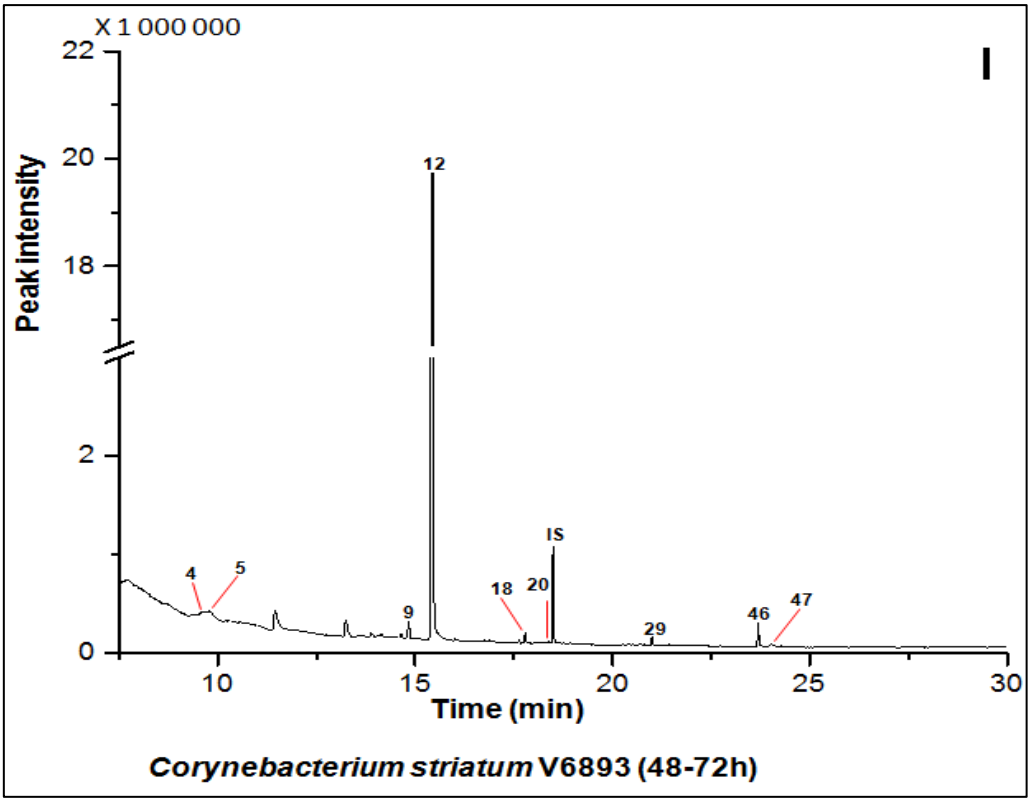
#University of Rostock: Medical Faculty, Institute of Medical Microbiology, Virology and Hygiene; ATCC: American Type Culture Collection; DSMZ: German Collection of Microorganisms and Cell Cultures; CCM: Czech Collection of Microorganisms; SCAM: Strain Collection of Antagonistic Microorganisms, Microbiology. *recently reclassified as *V. campbellii* ATCC BAA-1116.

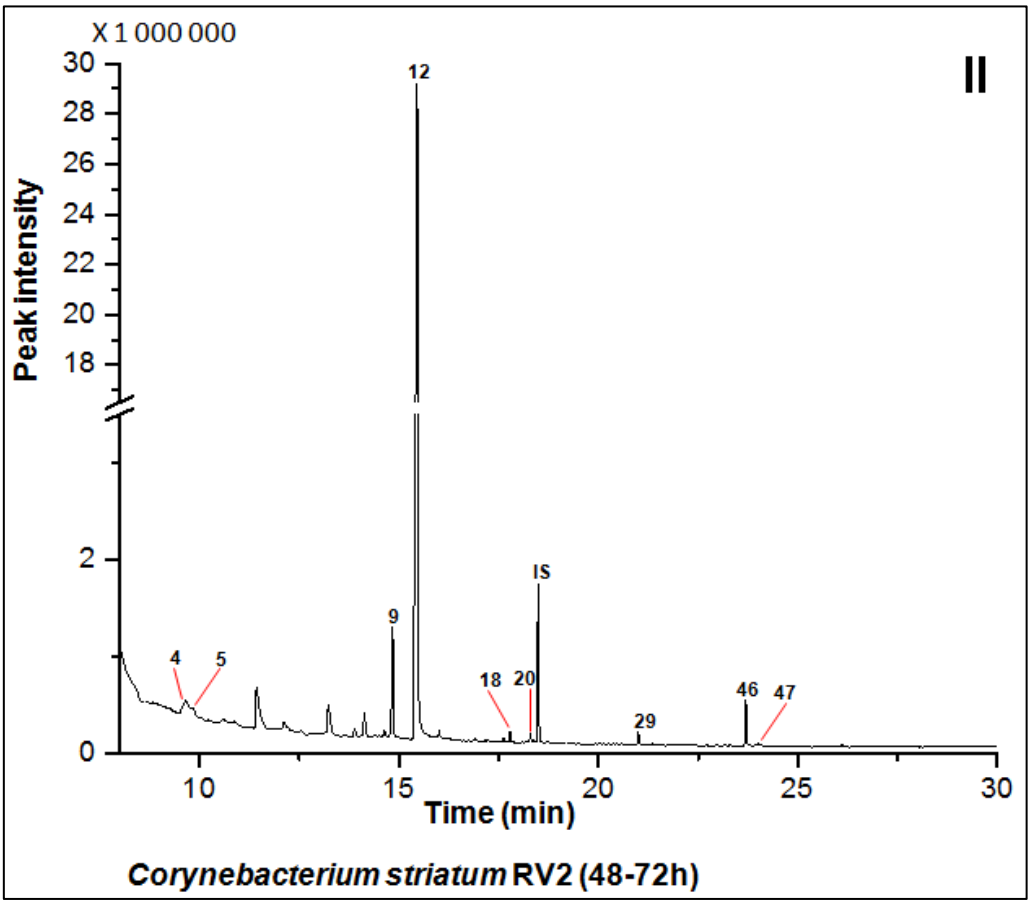
Table S4: Primers used in this study.

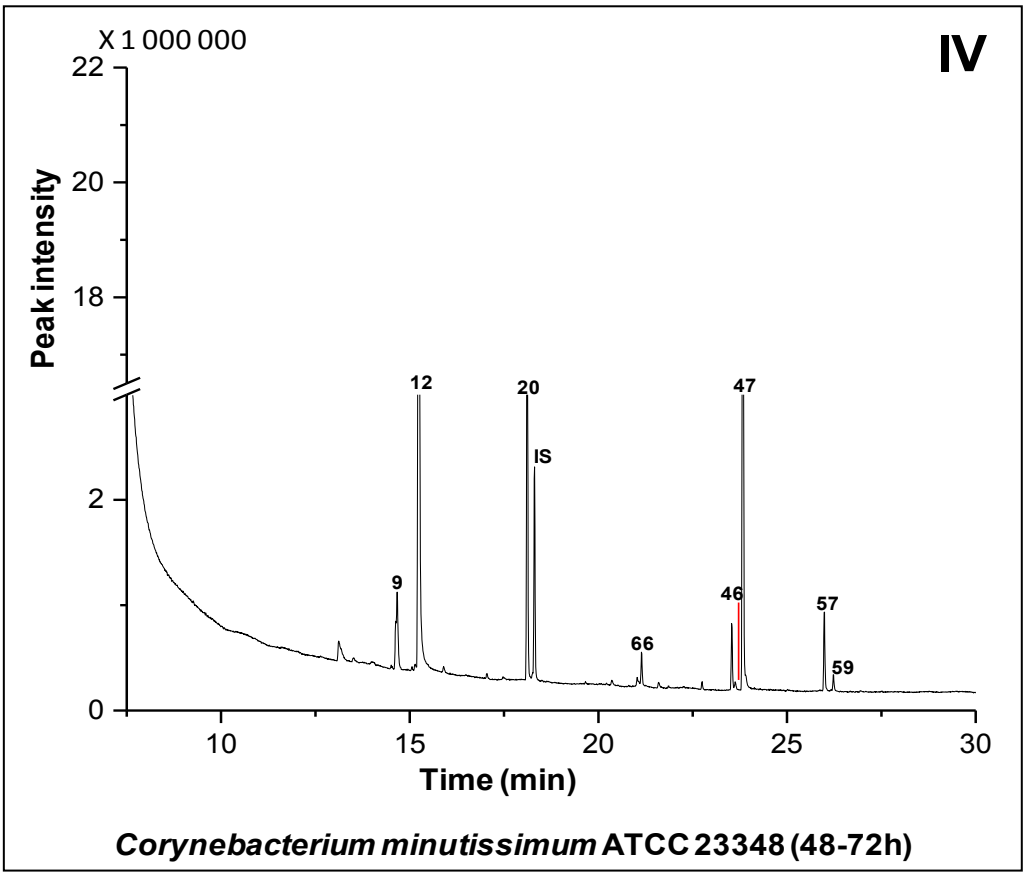
Gene Name	Direction	Tm (°C)	Sequence (5' ⟶ 3')	Use of primer
*SerAS9*_1741	Sense	60	TGCGTCTCTTGATTTCTCTC	RT-PCR Northern blot
	Antisense	60	TACTTTGCCAACTGCGCCTG	
*SerAS9*_1744	Sense	60	GAGTGCGGAGCAACTTCATC	RT-PCR Northern blot
	Antisense	60	GACCAGGCACGGCATCATAAC	
*SerAS9*_1745	Sense	60	ATAGGCTACCCACTGACAGG	RT-PCR
	Antisense	60	TGGATGCTGACGACCATGAC	
*SerAS9*_1747	Sense	60	CTGATACACCACATCGCCAC	RT-PCR Northern blot
	Antisense	60	ATACCTCACCTCCCAGCACC	
*SerAS9*_1749	Sense	60	TCTTCAGCAATTCAGGCACC	RT-PCR
	Antisense	60	CTGTGCCATAGGCTTTCTGC	RT
	Antisense	60	CAAATATCGGCCCGGCACAG	PCR
S*erAS9*_1750	Sense	60	AAAGCCCATCTTGCCGTCAG	RT-PCR Northern blot
	Antisense	60	GAATGCGTTCCTGGTAGGTG	RT Northern blot
	Antisense	60	GTGTTAATCACCGACAGACC	PCR
*SerAS9*_1751	Sense	60	CGACAACCTGCACTGAAAGC	RT-PCR Northern blot
	Antisense	60	ATATAGGCGCGGAACTGCTC	RT Northern blot
	Antisense	60	CGGAATGCTCGAAATCGAC	PCR
*SerAS9*_1752	Sense	60	TCTGCAACCACCGCGTAAAC	RT-PCR Northern blot
	Antisense	60	TACACCAGGAATACGGCTCG	
*SerAS9*_1753	Sense	60	GCCGGTTCCGATATTTATGC	RT-PCR
	Antisense	60	TAGAGCATTAGCCGACACTG	RT
	Antisense	60	GTTCCAGACGCAGTTTCATC	PCR

Table S4: Primers used in this study (cont.).

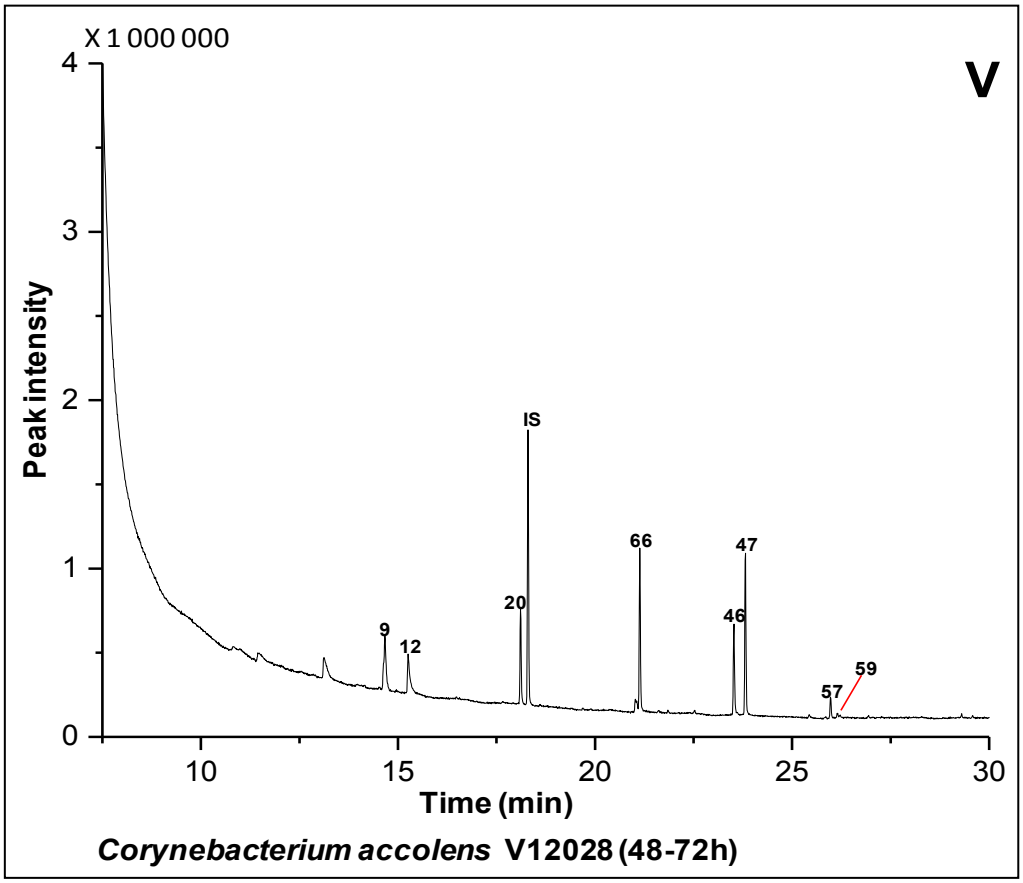
*SerAS9*_0621	Sense	60	ATGGACATCAATCTGCCAGG	RT-PCR
	Antisense	60	CGATGATTTCCGGCGTATCC	
*SerAS9*_4328	Sense	60	GCATCCTTCTCTAACCGCAC	RT-PCR
	Antisense	60	ATCGCACGTTATCCTCCATG	Northern blot
*SerAS9*_1501	Sense	60	CATGTTGTGCCTGCTGATGG	RT-PCR
	Antisense	60	TCCAGTCGCGCTCGATAAAG	Northern blot
*SerAS9*_0772	Sense	60	CCACTGCTGGATAGCTTCAC	RT-PCR
	Antisense	60	CCAGCTCTTCGTTGTGGTTC	Northern blot
*SerAS9*_0078	Sense	60	CGCTTCGGGAGCATATCGAC	RT-PCR Northern blot


X 1 000 000
I
Peak intensity
Time (min)
12
IS
4
5
9
18
20
29
46
47
Corynebacterium striatum V6893 (48-72h)

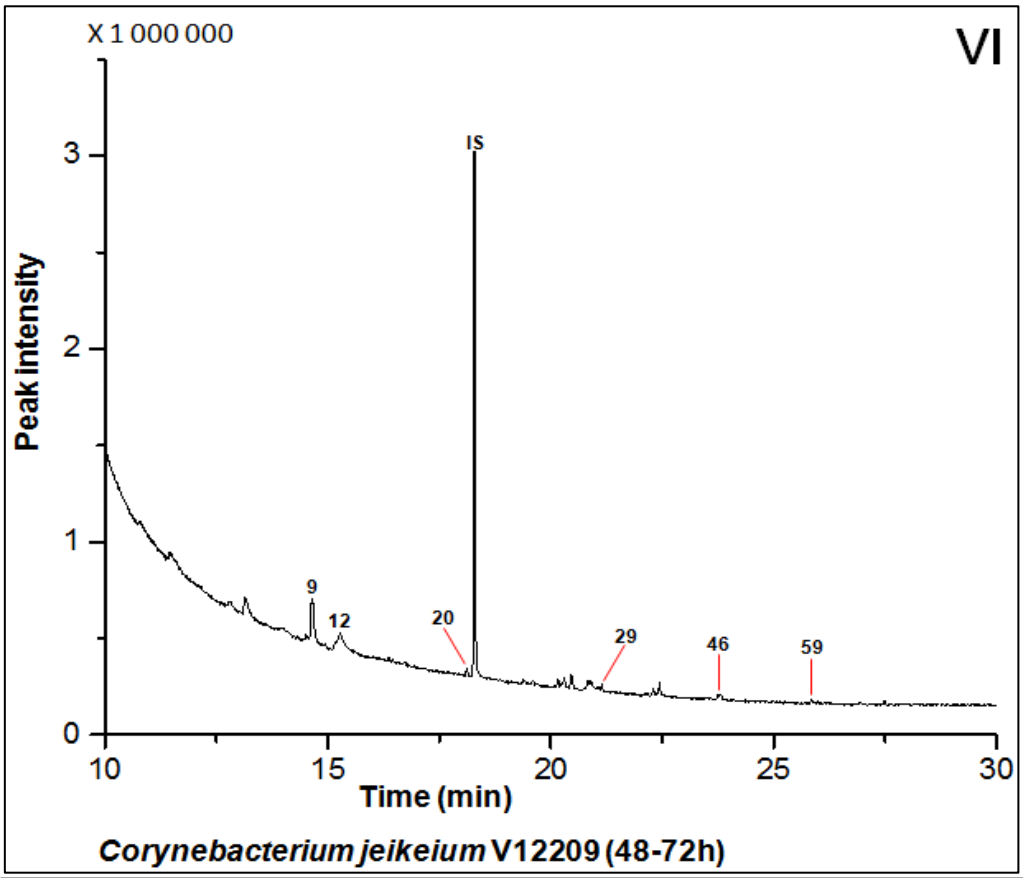

X 1 000 000
II
Peak intensity
Time (min)
12
IS
4
5
9
18
20
29
46
47
Corynebacterium striatum RV2 (48-72h)

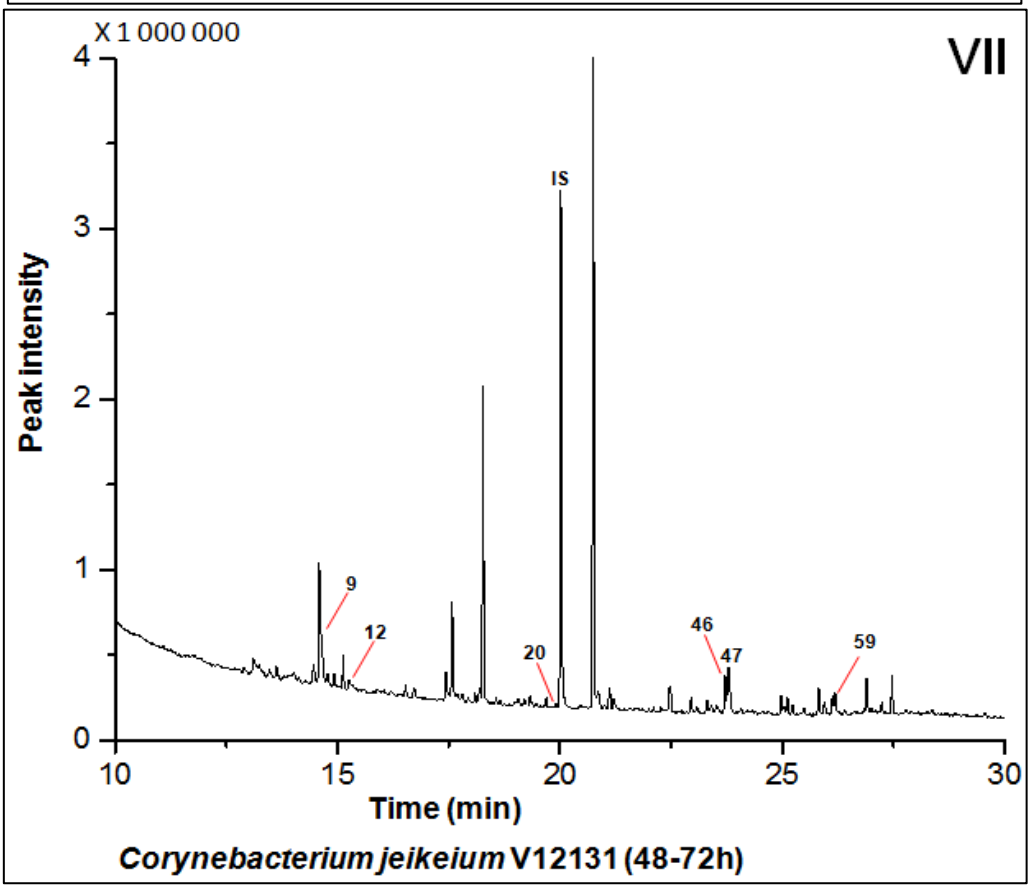


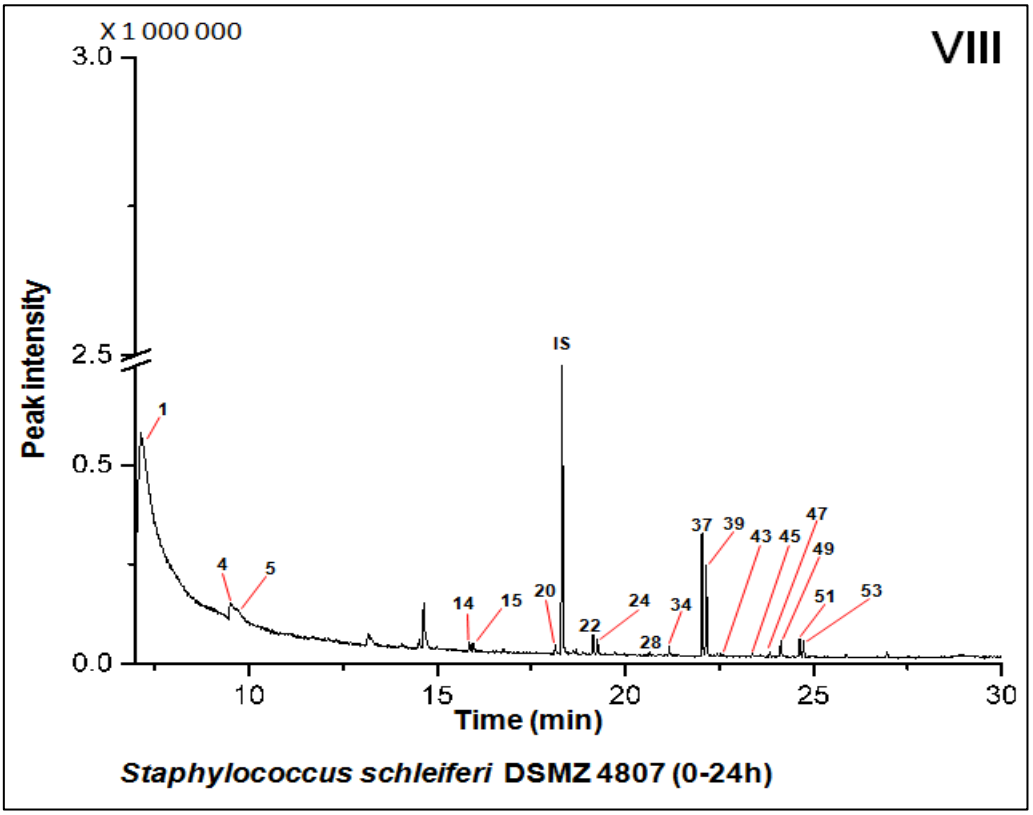
Corynebacterium minutissimum ATCC 23348 (48-72h)

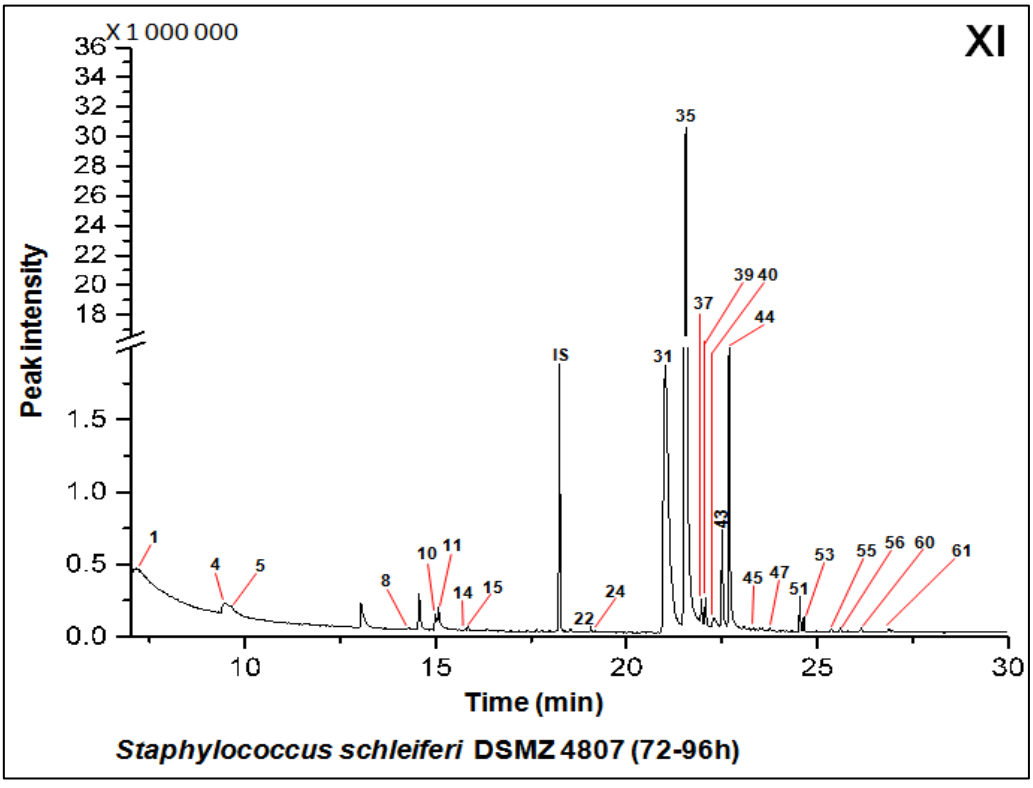


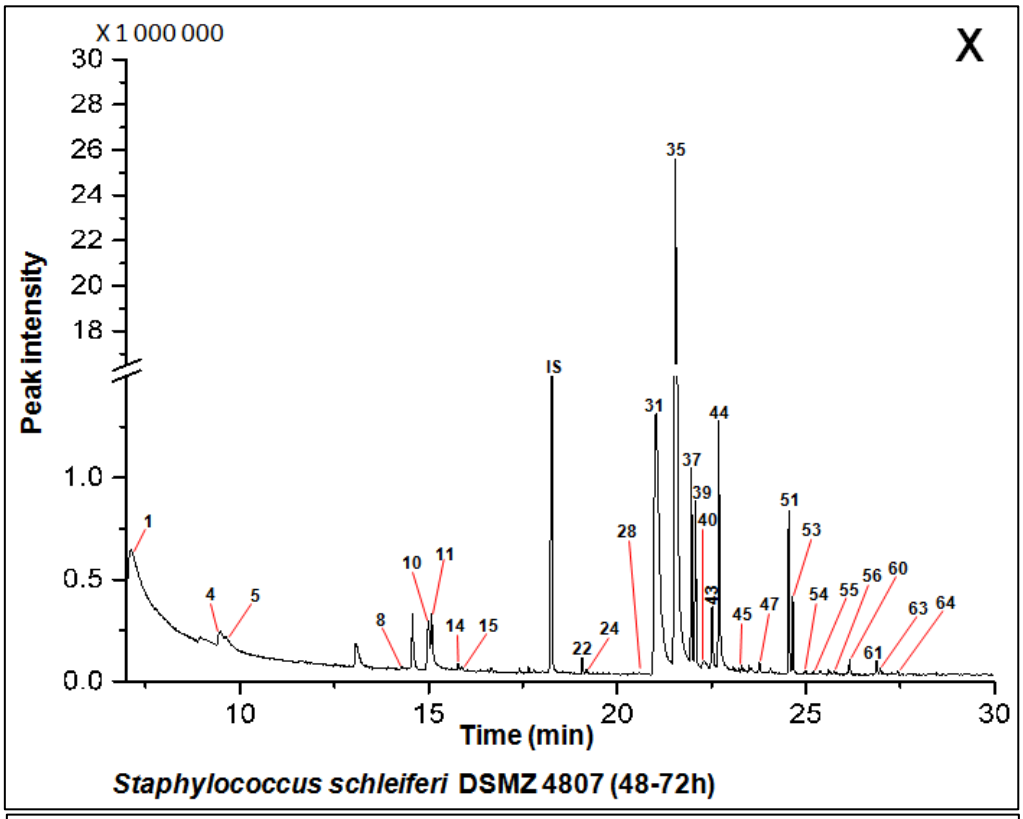
Corynebacterium accolens V12028 (48-72h)

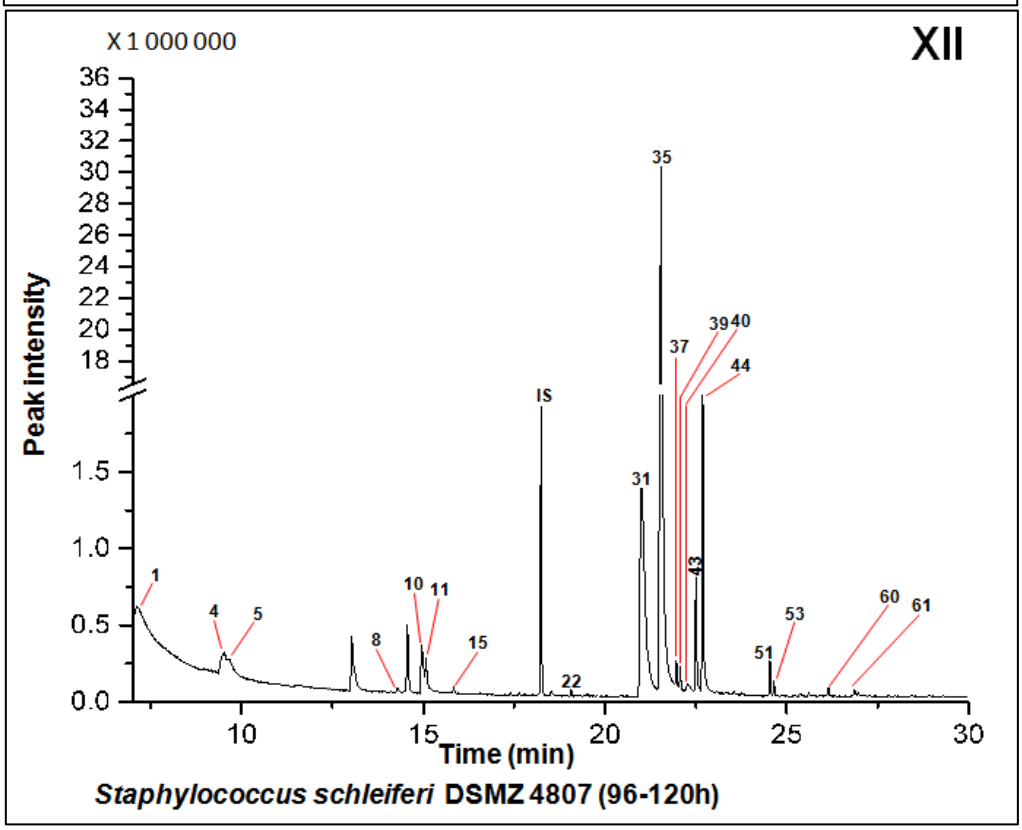

X 1 000 000
VI
Peak intensity
IS
9
12
20
29
46
59
Time (min)
Corynebacterium jeikeium V12209 (48-72h)

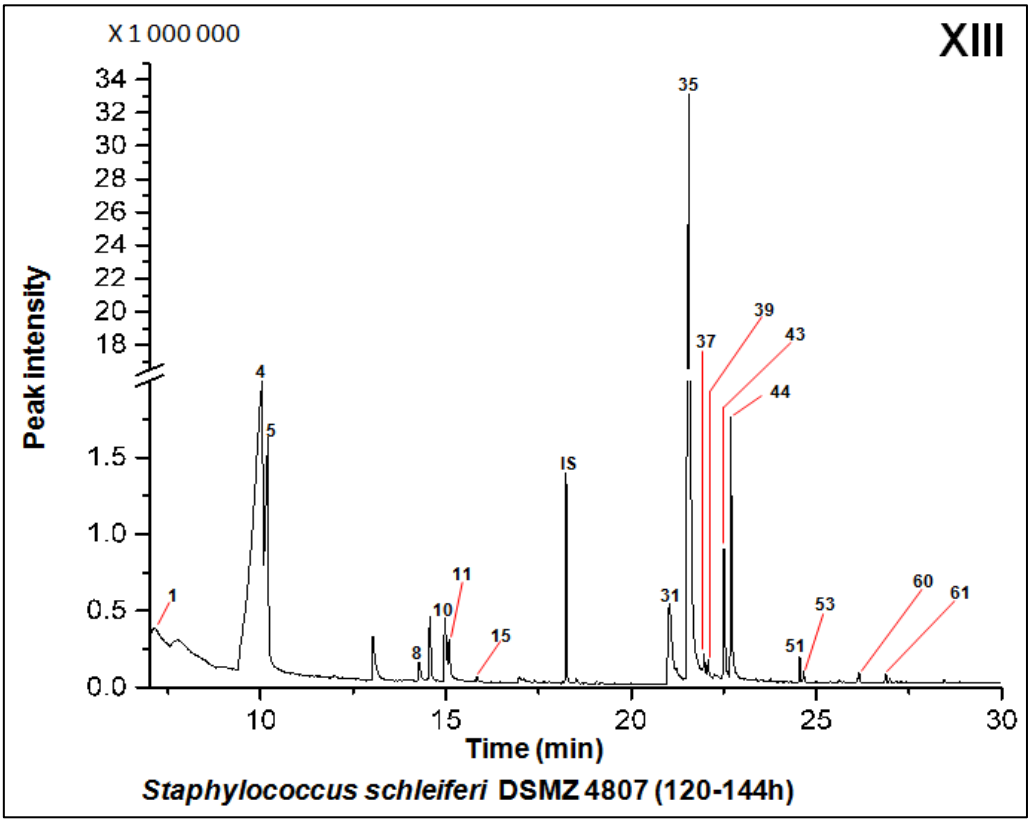

X 1 000 000
VII
Peak intensity
IS
9
12
20
46
47
59
Time (min)
Corynebacterium jeikeium V12131 (48-72h)

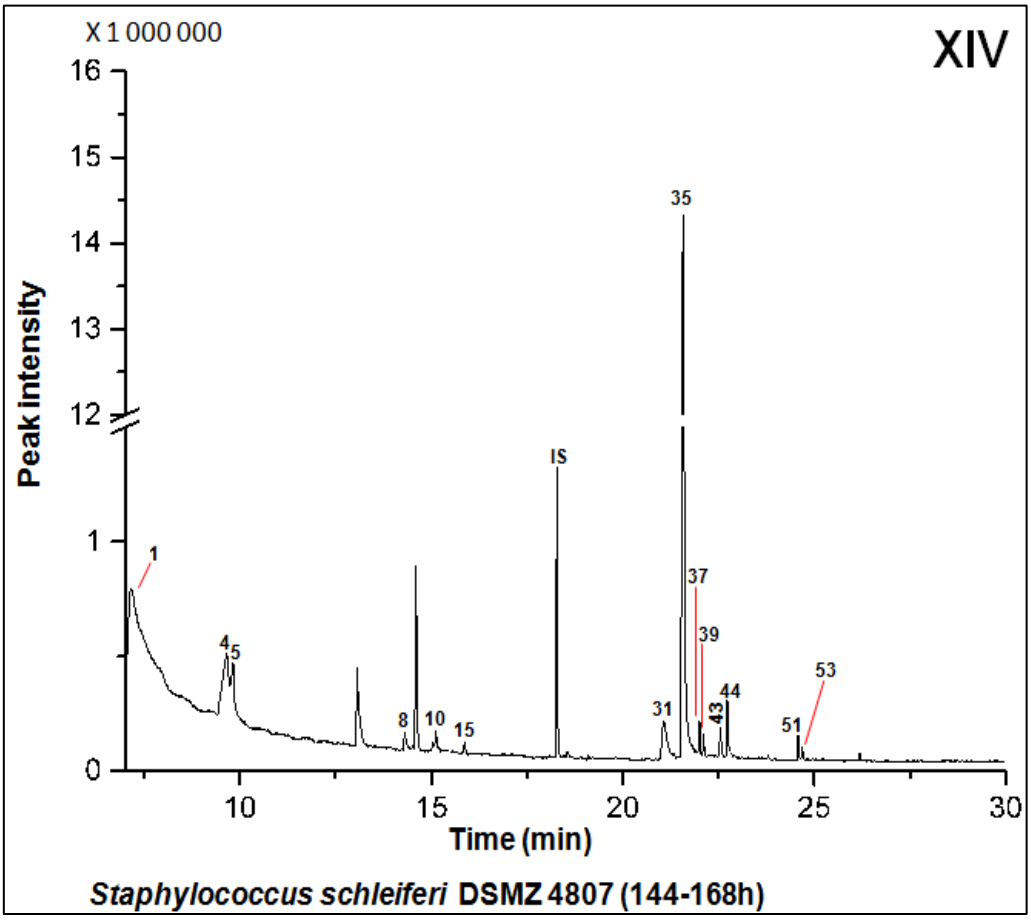

X 1 000 000
VIII
Peak intensity
Time (min)
IS
1
4
5
14
15
20
22
24
28
34
37
39
43
45
47
49
51
53
Staphylococcus schleiferi DSMZ 4807 (0-24h)

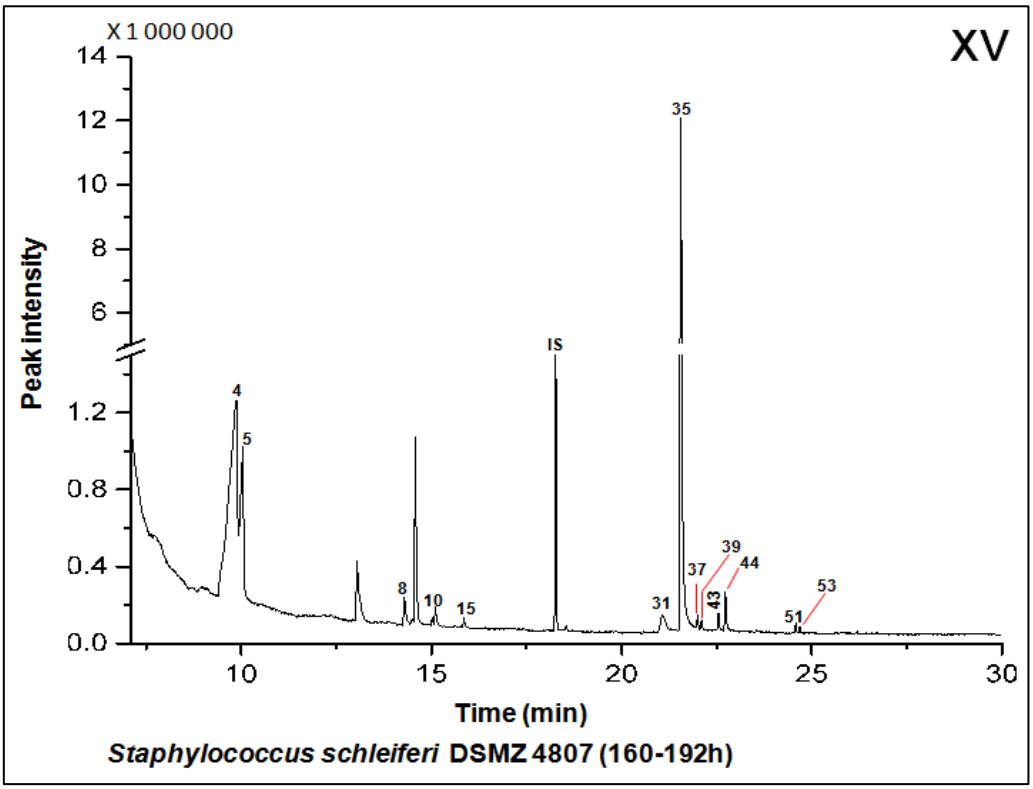

X 1 000 000
XI
Peak intensity
Time (min)
IS
1
4
5
8
10
11
14
15
22
24
31
35
37
39
40
43
44
45
47
51
53
55
56
60
61
Staphylococcus schleiferi DSMZ 4807 (72-96h)

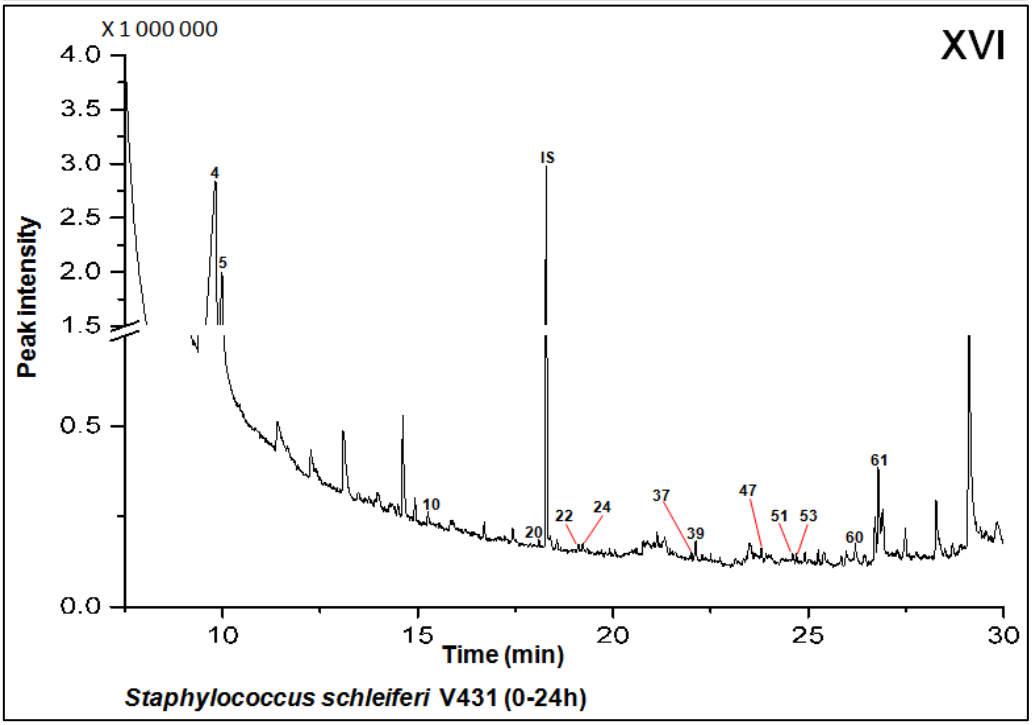

X
X 1 000 000
Peak intensity
Time (min)
IS
Staphylococcus schleiferi DSMZ 4807 (48-72h)

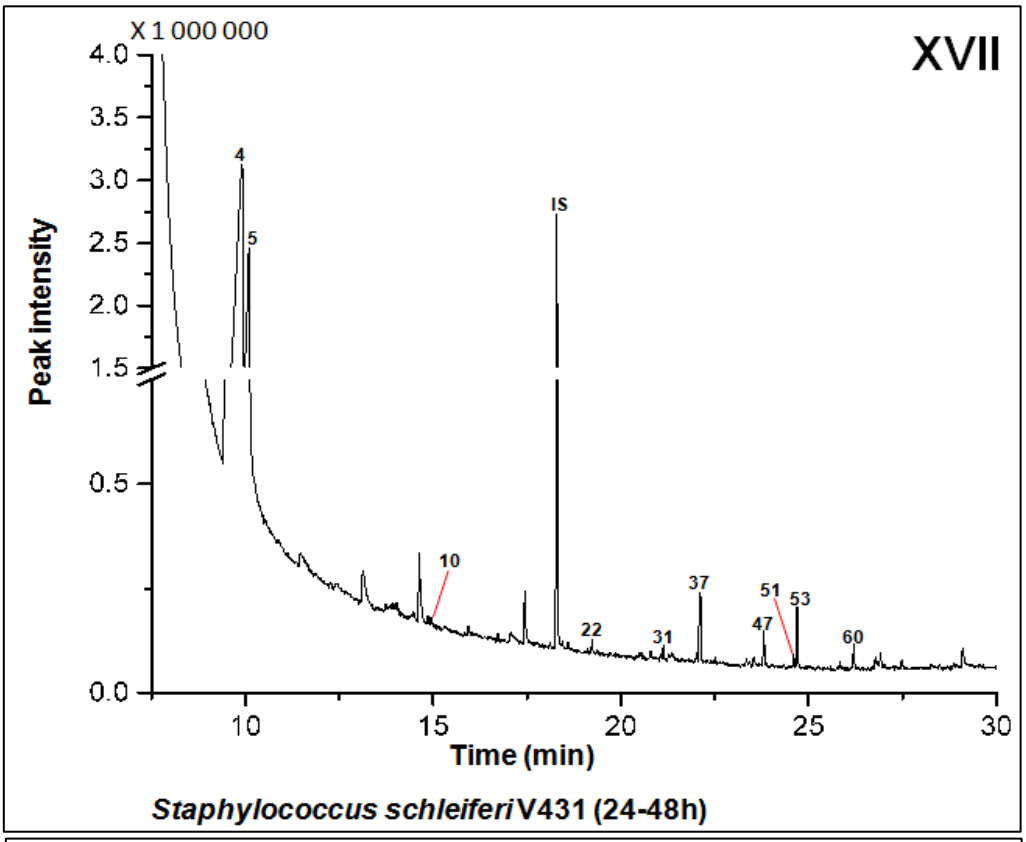

XII
X 1 000 000
Peak intensity
Time (min)
IS
Staphylococcus schleiferi DSMZ 4807 (96-120h)

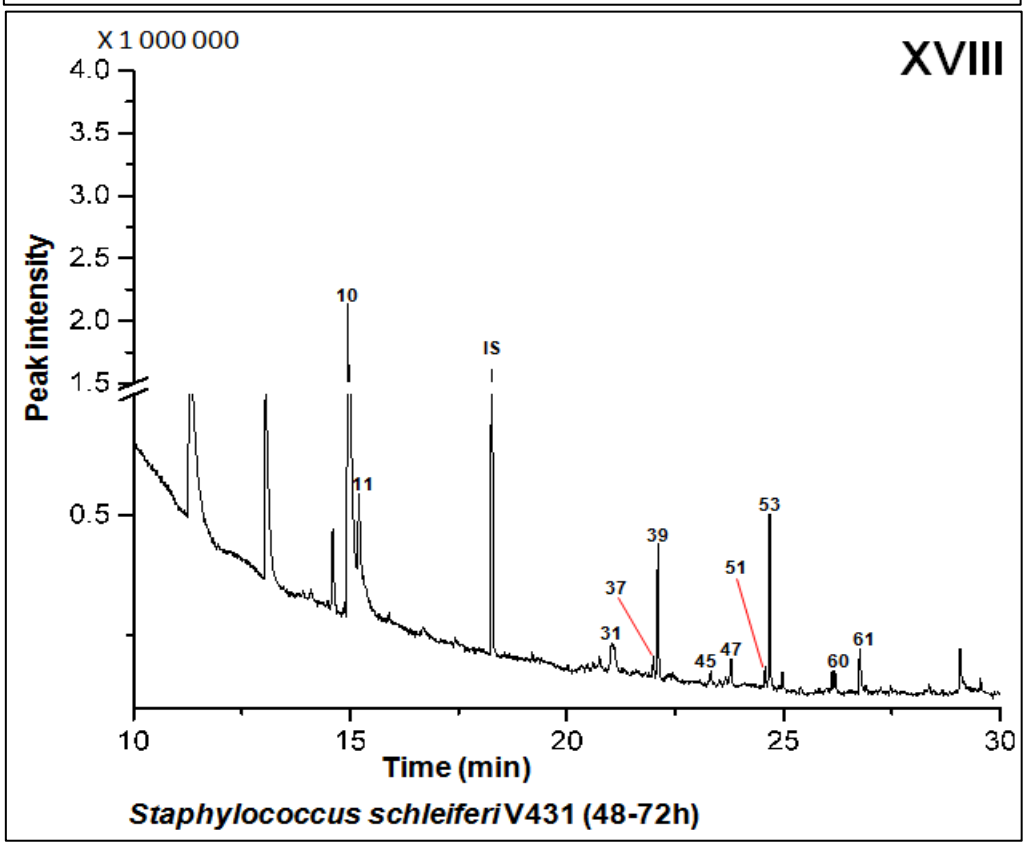

X 1 000 000
XIII
Peak intensity
Time (min)
IS
Staphylococcus schleiferi DSMZ 4807 (120-144h)

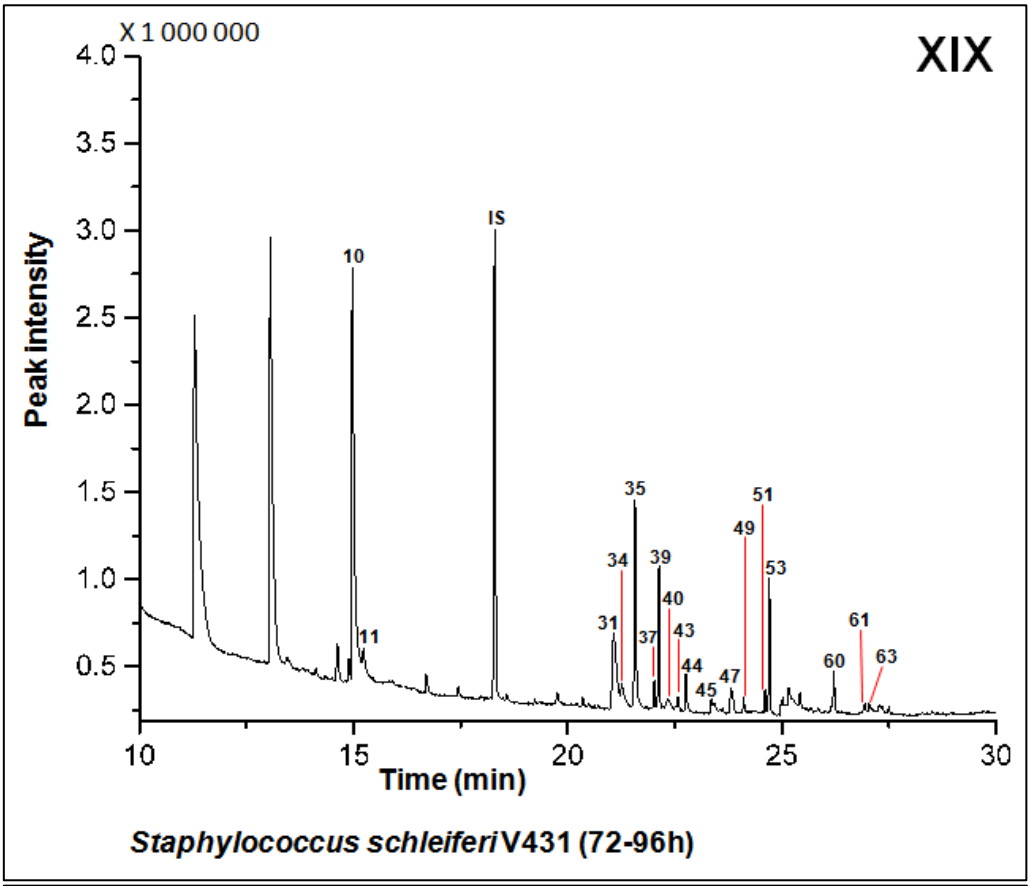

X 1 000 000
XIV
Peak intensity
Time (min)
IS
Staphylococcus schleiferi DSMZ 4807 (144-168h)

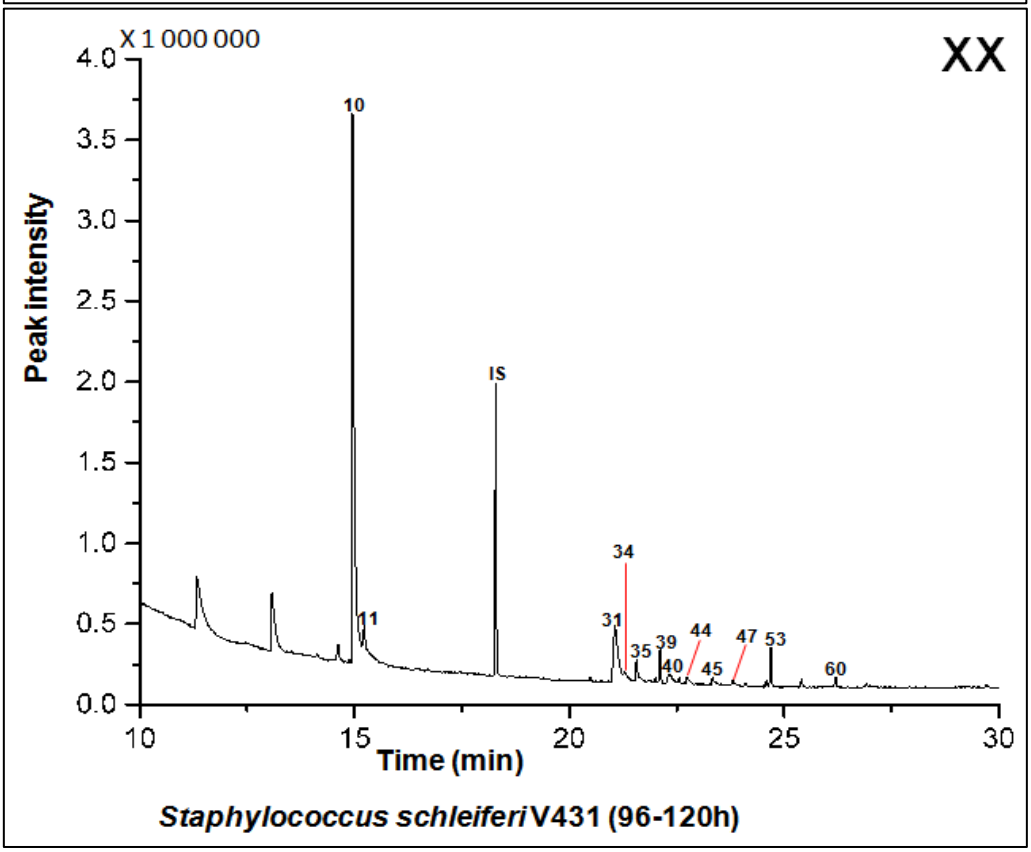

X 1 000 000
XV
Peak intensity
Time (min)
IS
4
5
8
10
15
31
35
37
39
43
44
51
53
Staphylococcus schleiferi DSMZ 4807 (160-192h)

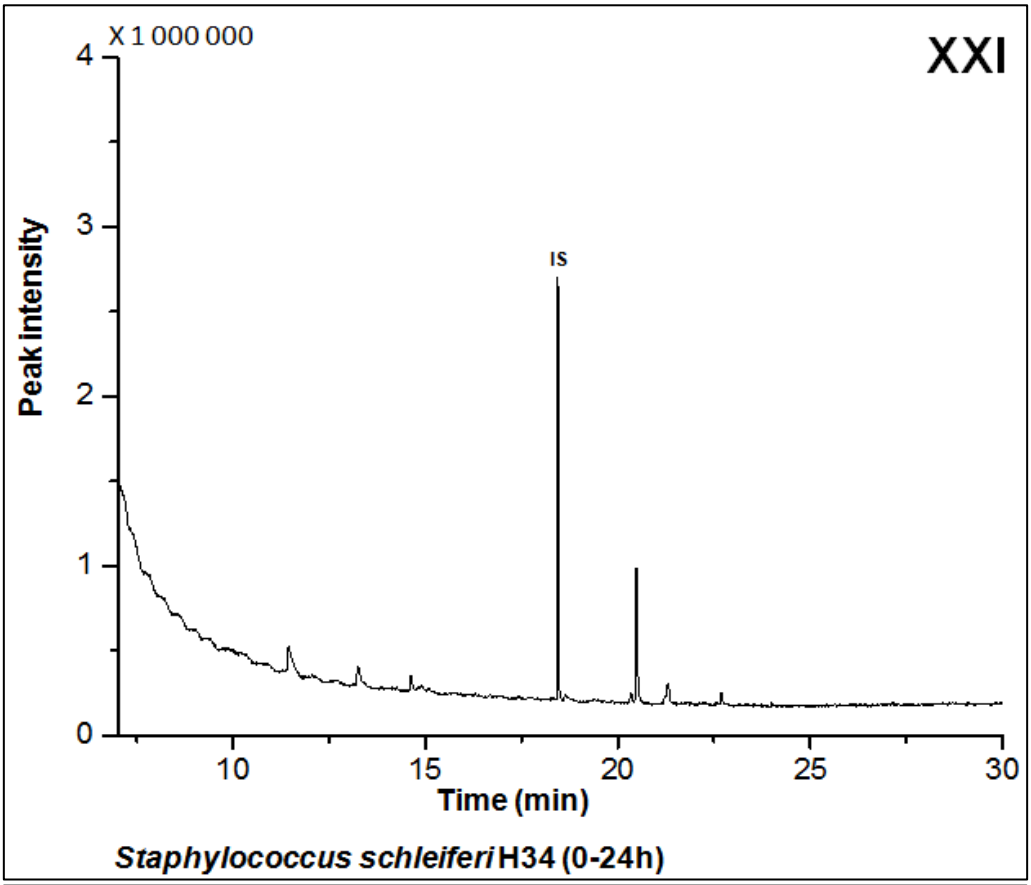

X 1 000 000
XVI
Peak intensity
Time (min)
IS
4
5
10
20
22
24
37
39
47
51
53
60
61
Staphylococcus schleiferi V431 (0-24h)

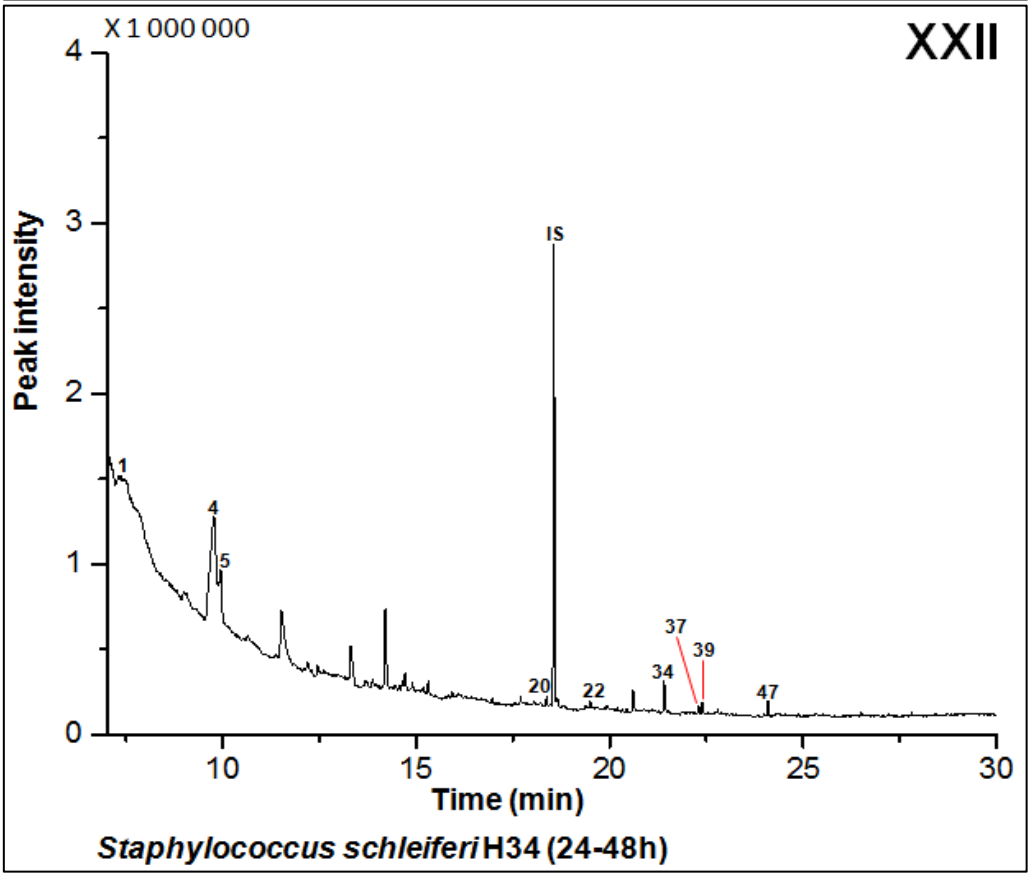

XVII
X 1 000 000
Peak intensity
Time (min)
4
5
IS
10
22
31
37
47
51
53
60
Staphylococcus schleiferi V431 (24-48h)

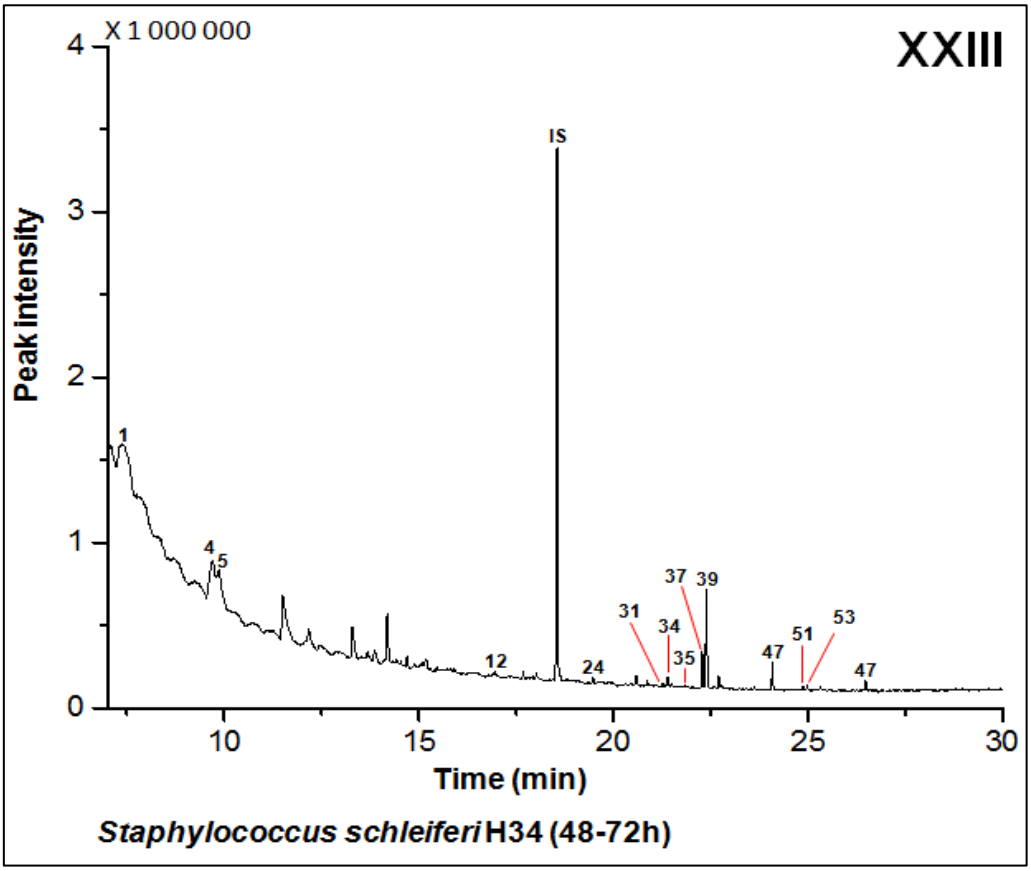

XVIII
X 1 000 000
Peak intensity
Time (min)
10
11
IS
31
37
39
45
47
51
53
60
61
Staphylococcus schleiferi V431 (48-72h)

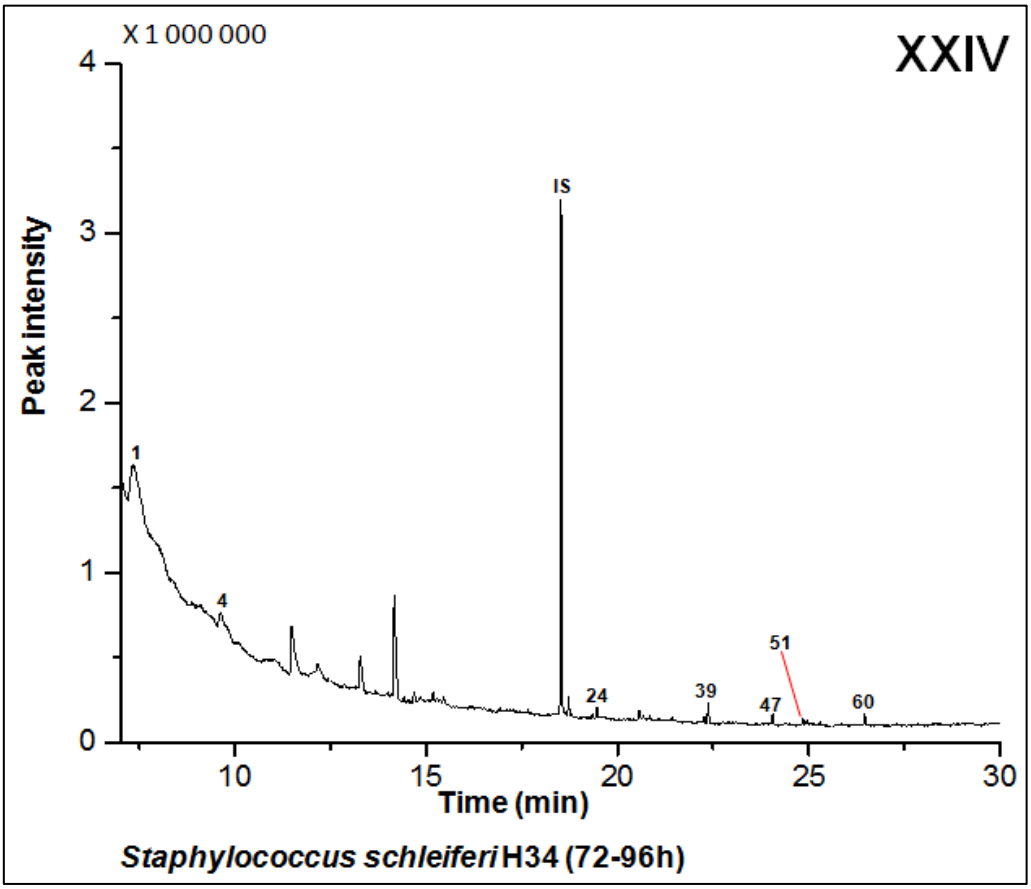

XIX
X 1 000 000
Peak intensity
Time (min)
IS
10
11
31
34
35
37
39
40
43
44
45
47
49
51
53
60
61
63
Staphylococcus schleiferi V431 (72-96h)

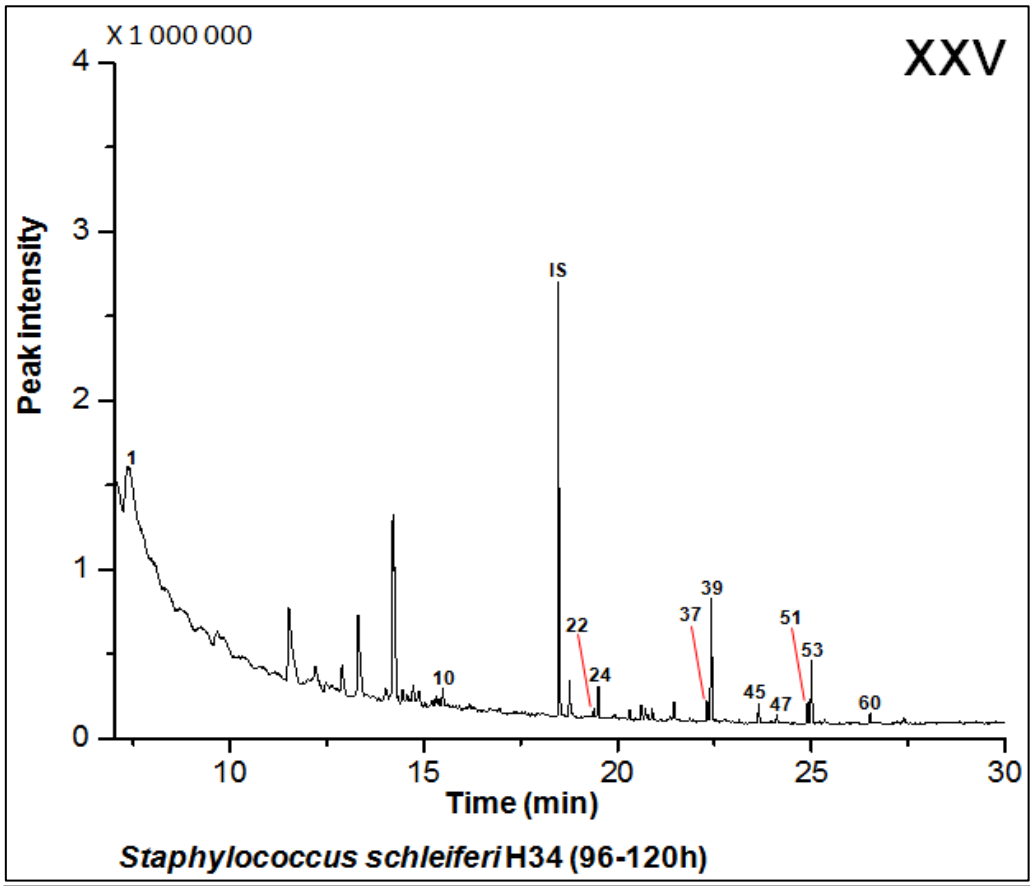

XX
X 1 000 000
Peak intensity
Time (min)
IS
10
11
31
34
35
39
40
44
45
47
53
60
Staphylococcus schleiferi V431 (96-120h)

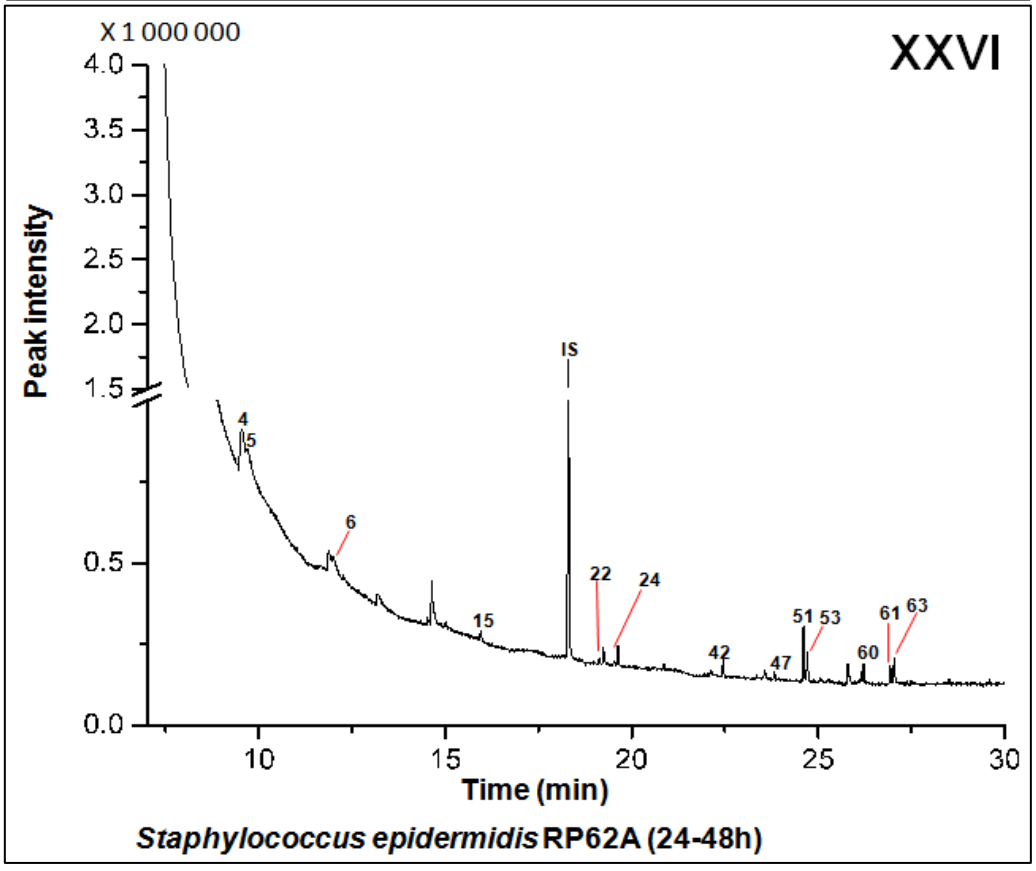

X 1 000 000
XXI
Peak intensity
IS
Time (min)
Staphylococcus schleiferi H34 (0-24h)

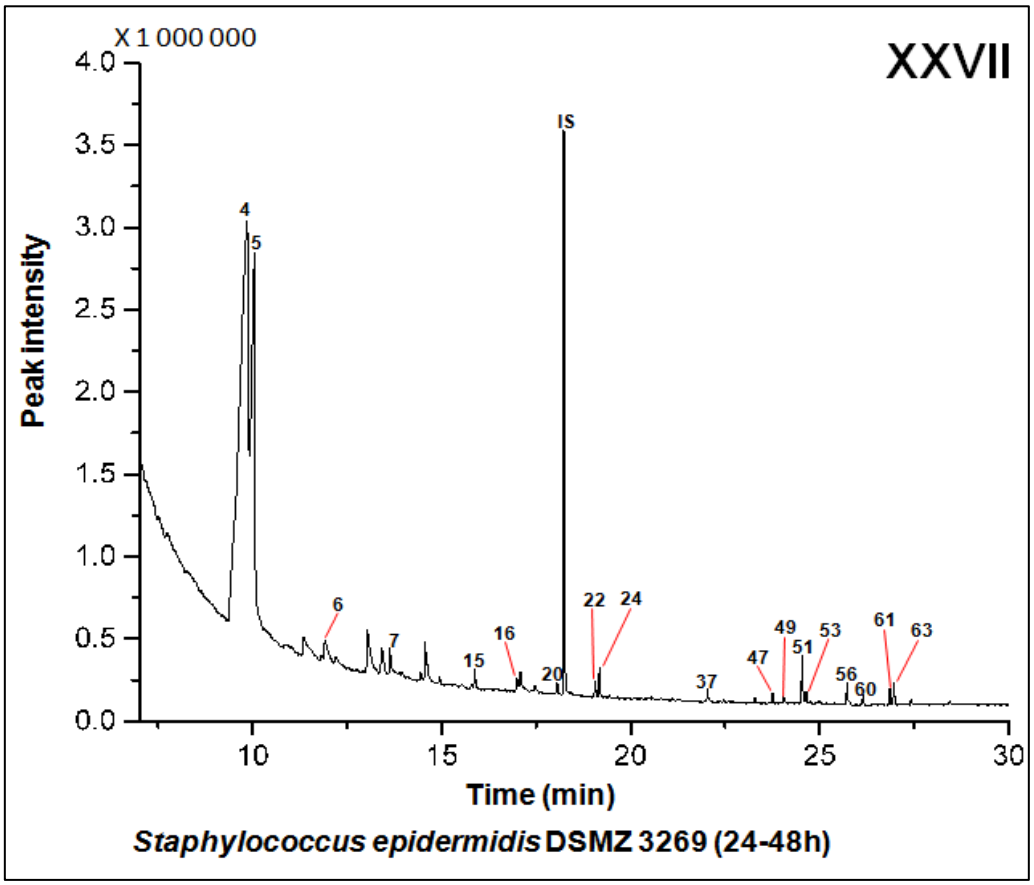

X 1 000 000
XXII
Peak intensity
IS
1
4
5
20
22
34
37
39
47
Time (min)
Staphylococcus schleiferi H34 (24-48h)

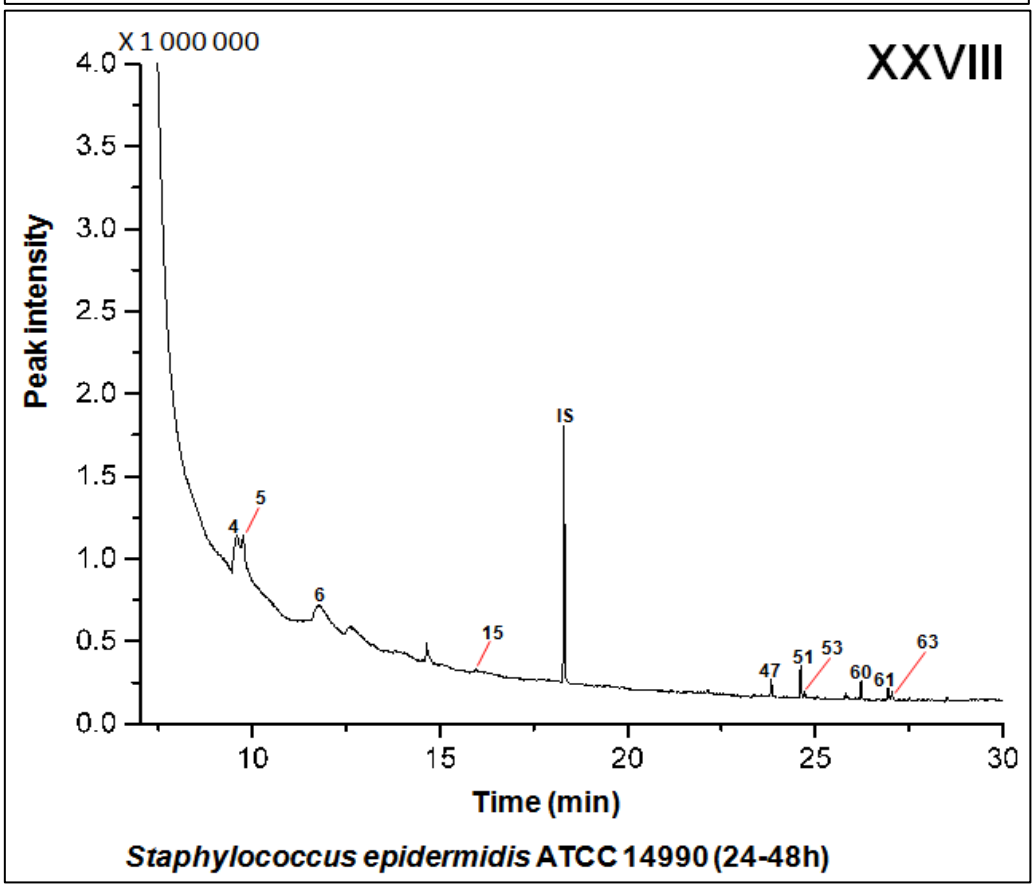

XXIII
X 1 000 000
Peak intensity
Time (min)
IS
1
4
5
12
24
31
34
35
37
39
47
51
53
47
Staphylococcus schleiferi H34 (48-72h)

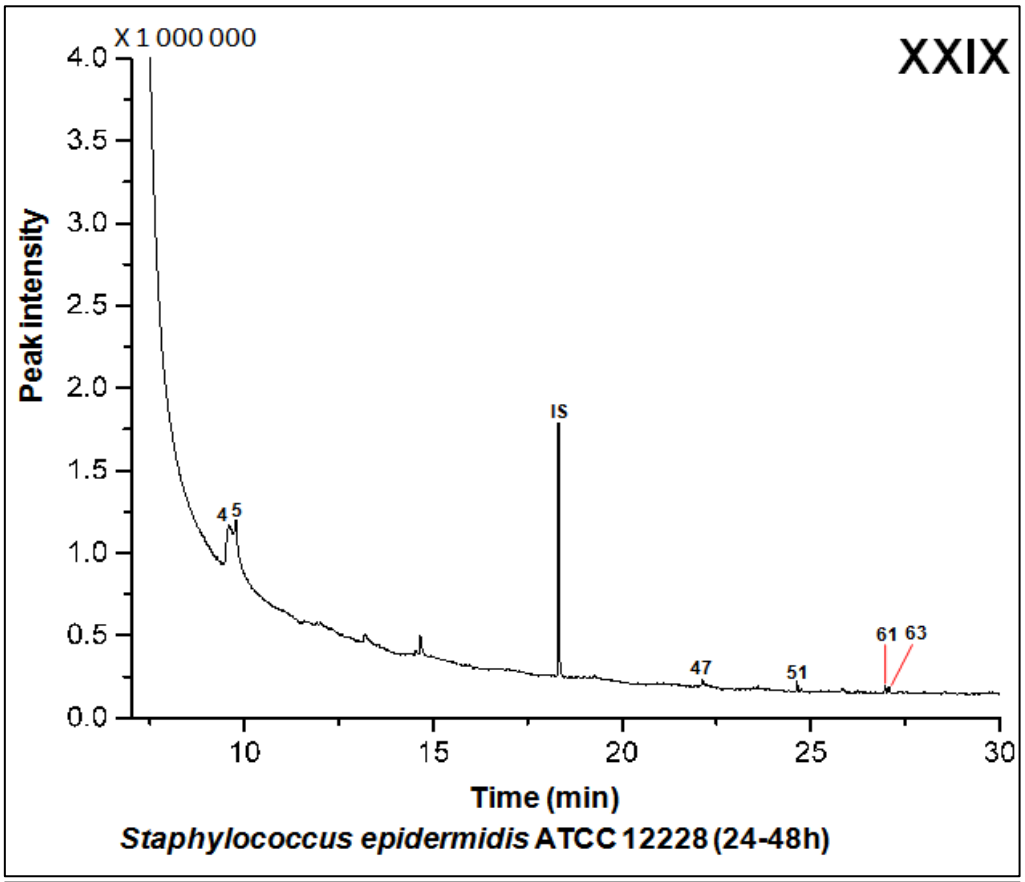

XXIV
X 1 000 000
Peak intensity
Time (min)
IS
1
4
24
39
47
51
60
Staphylococcus schleiferi H34 (72-96h)

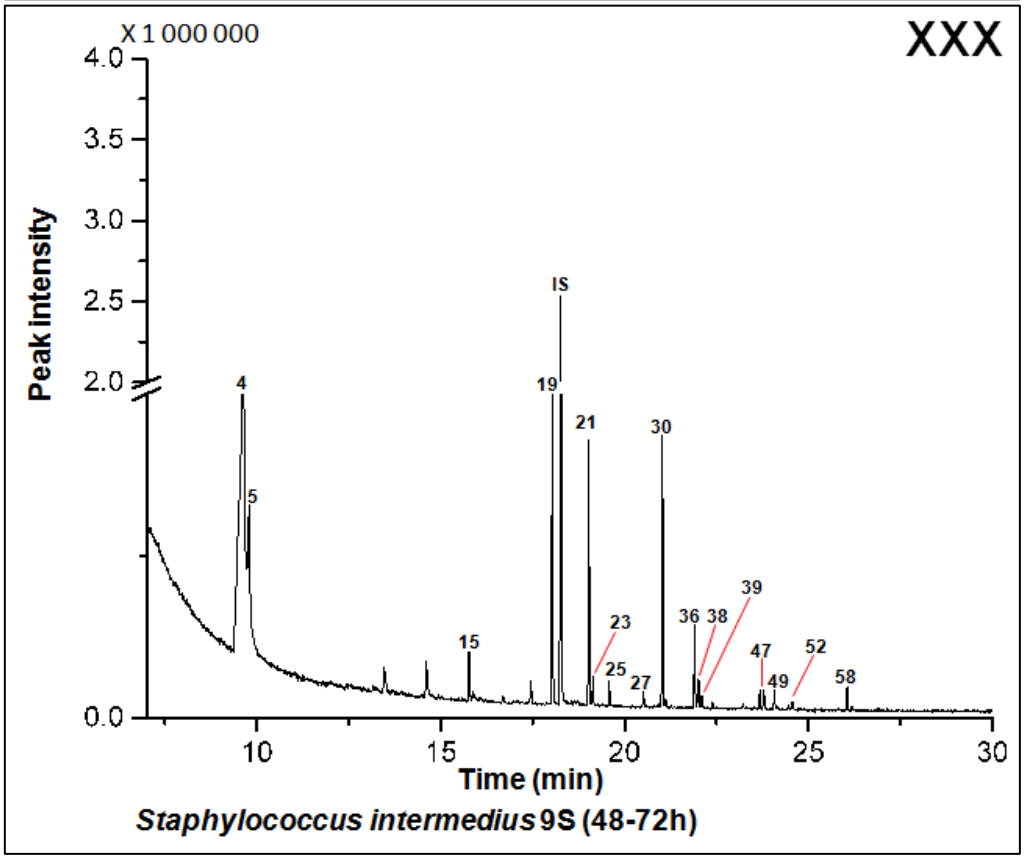

X 1 000 000
XXV
Peak intensity
Time (min)
IS
1
10
22
24
37
39
45
47
51
53
60
Staphylococcus schleiferi H34 (96-120h)

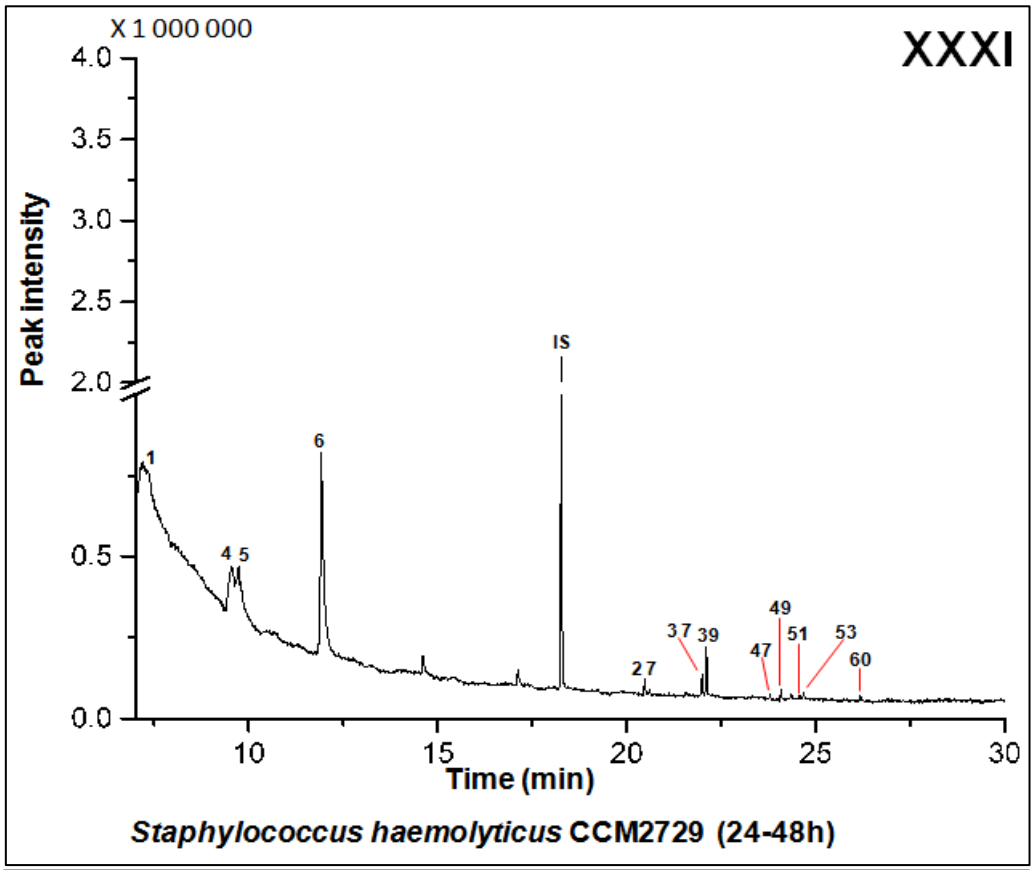

X 1 000 000
XXVI
Peak intensity
Time (min)
IS
4
5
6
15
22
24
42
47
51
53
60
61
63
Staphylococcus epidermidis RP62A (24-48h)

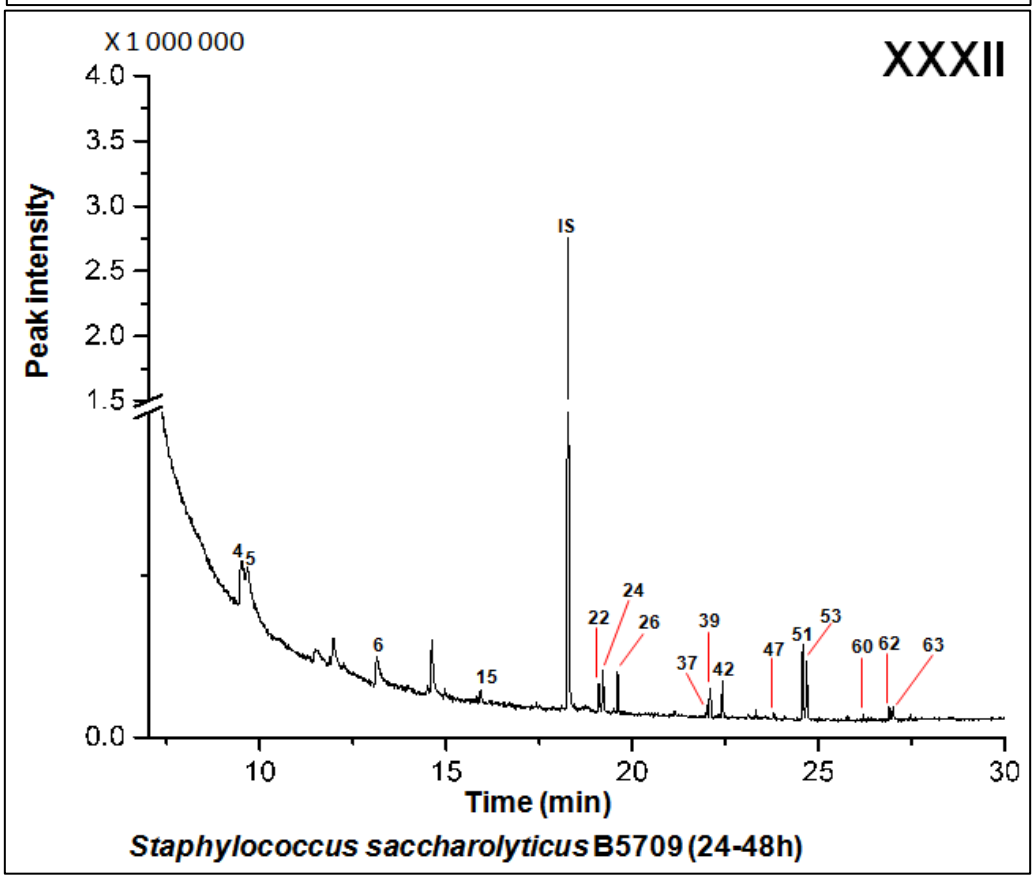

XXVII
X 1 000 000
Peak intensity
Time (min)
IS
4
5
6
7
15
16
20
22
24
37
47
49
51
53
56
60
61
63
Staphylococcus epidermidis DSMZ 3269 (24-48h)

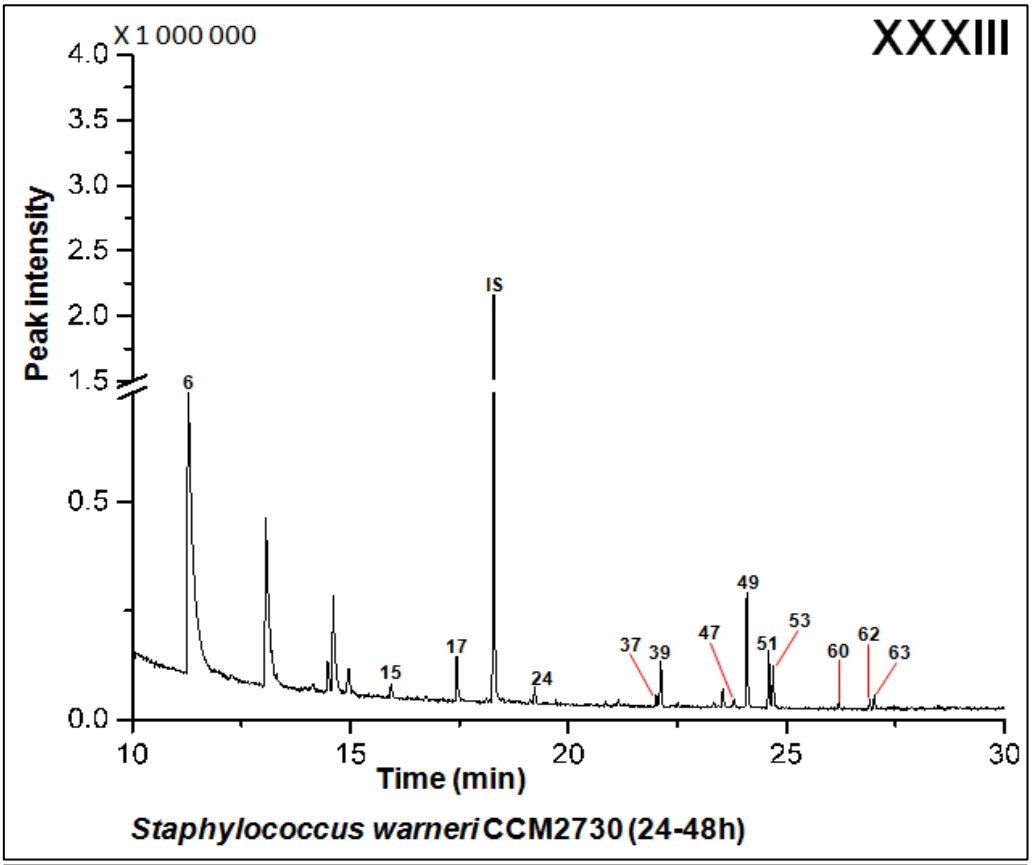

XXVIII
X 1 000 000
Peak intensity
Time (min)
IS
4
5
6
15
47
51
53
60
61
63
Staphylococcus epidermidis ATCC 14990 (24-48h)

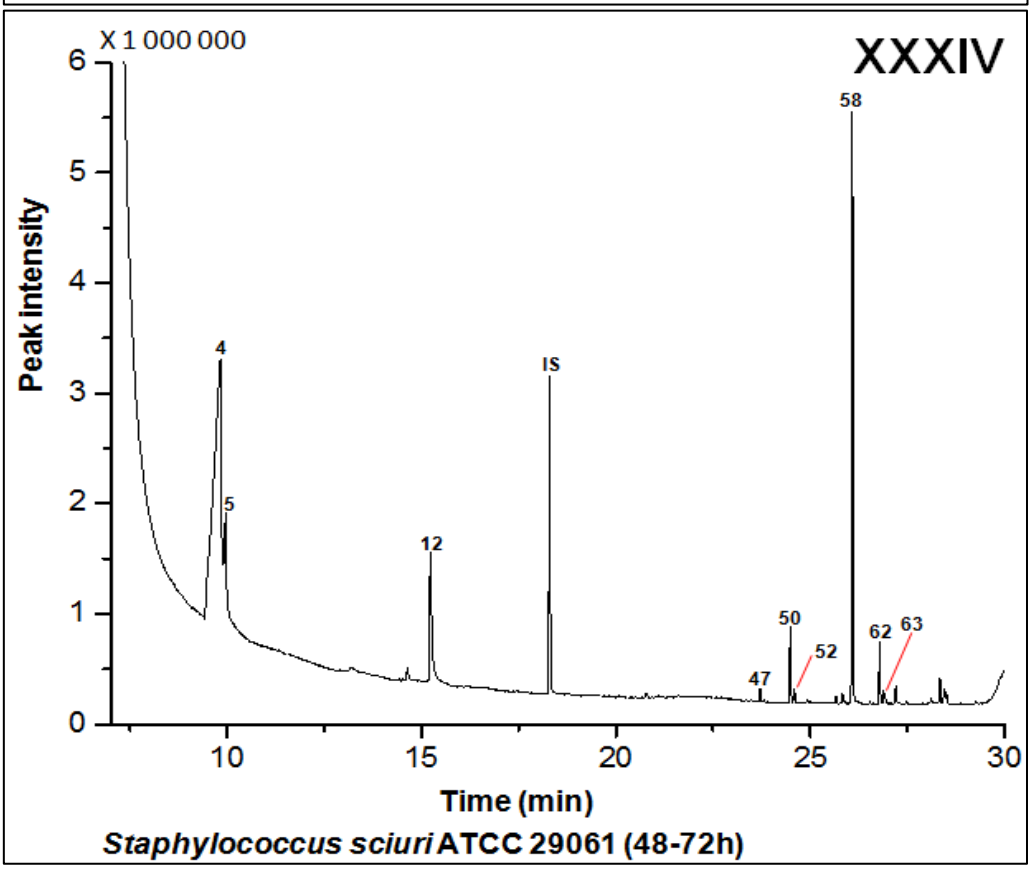

XXIX
X 1 000 000
Peak intensity
Time (min)
IS
4
5
47
51
61
63
Staphylococcus epidermidis ATCC 12228 (24-48h)

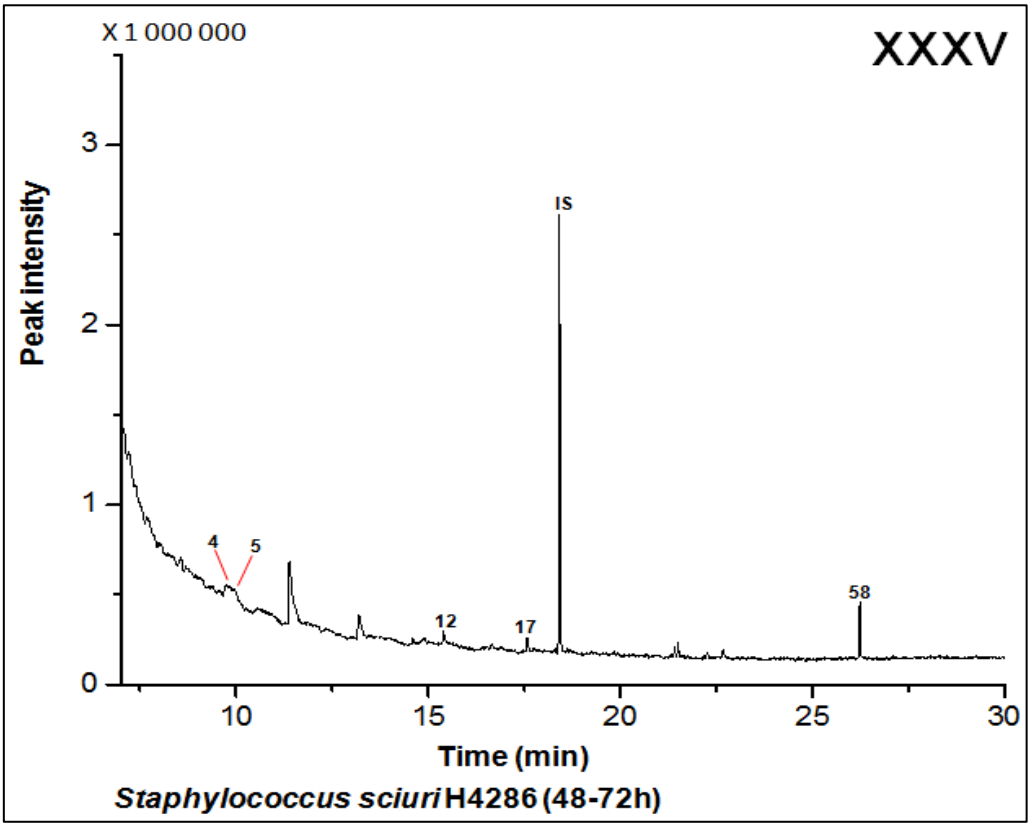

XXX
X 1 000 000
Peak intensity
Time (min)
IS
4
5
19
21
30
15
23
25
27
36
38
39
47
49
52
58
Staphylococcus intermedius 9S (48-72h)

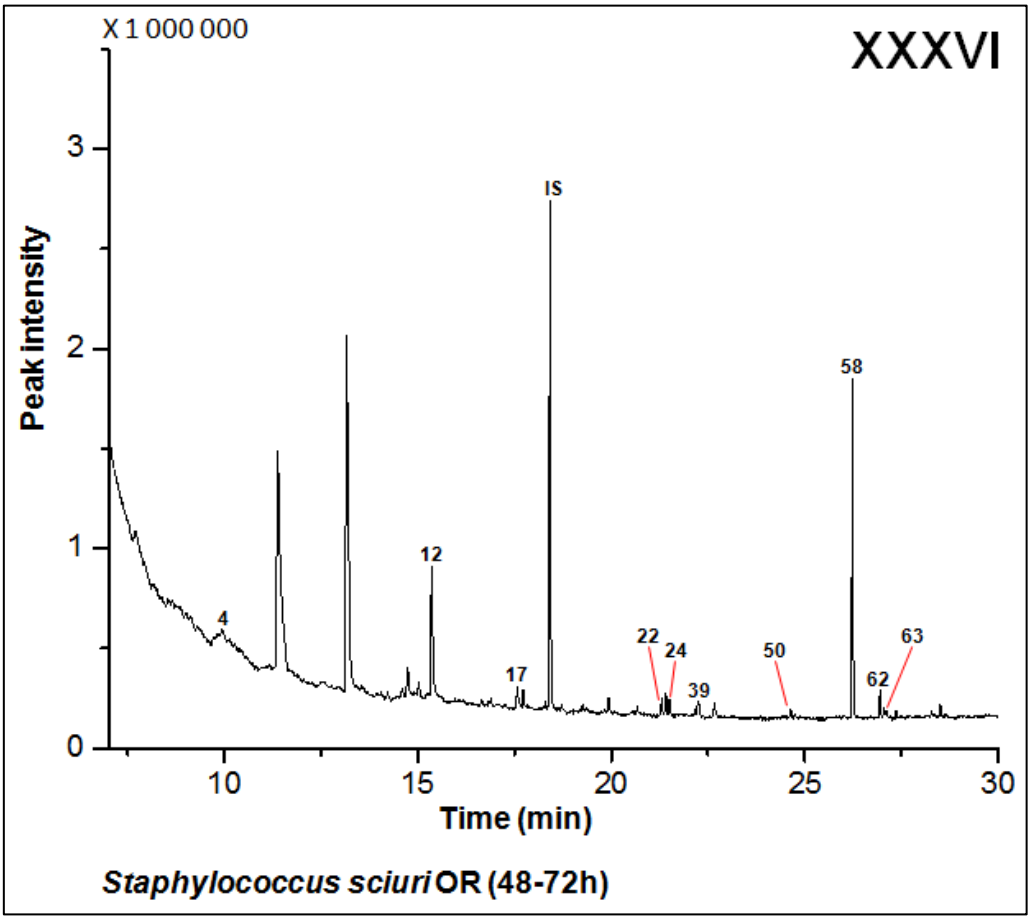

X 1 000 000
XXXI
Peak intensity
Time (min)
IS
1
4 5
6
27
37 39
47
49
51
53
60
Staphylococcus haemolyticus CCM2729 (24-48h)


X 1 000 000
XXXII
Peak intensity
Time (min)
IS
4 5
6
15
22
24
26
37
39
42
47
51
53
60
62
63
Staphylococcus saccharolyticus B5709 (24-48h)


XXXIII
X 1 000 000
Peak intensity
Time (min)
IS
6
15
17
24
37
39
47
49
51
53
60
62
63
Staphylococcus warneri CCM2730 (24-48h)


XXXIV
X 1 000 000
Peak intensity
Time (min)
IS
4
5
12
47
50
52
58
62
63
Staphylococcus sciuri ATCC 29061 (48-72h)


XXXV
X 1 000 000
Peak intensity
Time (min)
IS
4
5
12
17
58
Staphylococcus sciuri H4286 (48-72h)


XXXVI
X 1 000 000
Peak intensity
Time (min)
IS
4
12
17
22
24
39
50
58
62
63
Staphylococcus sciuri OR (48-72h)

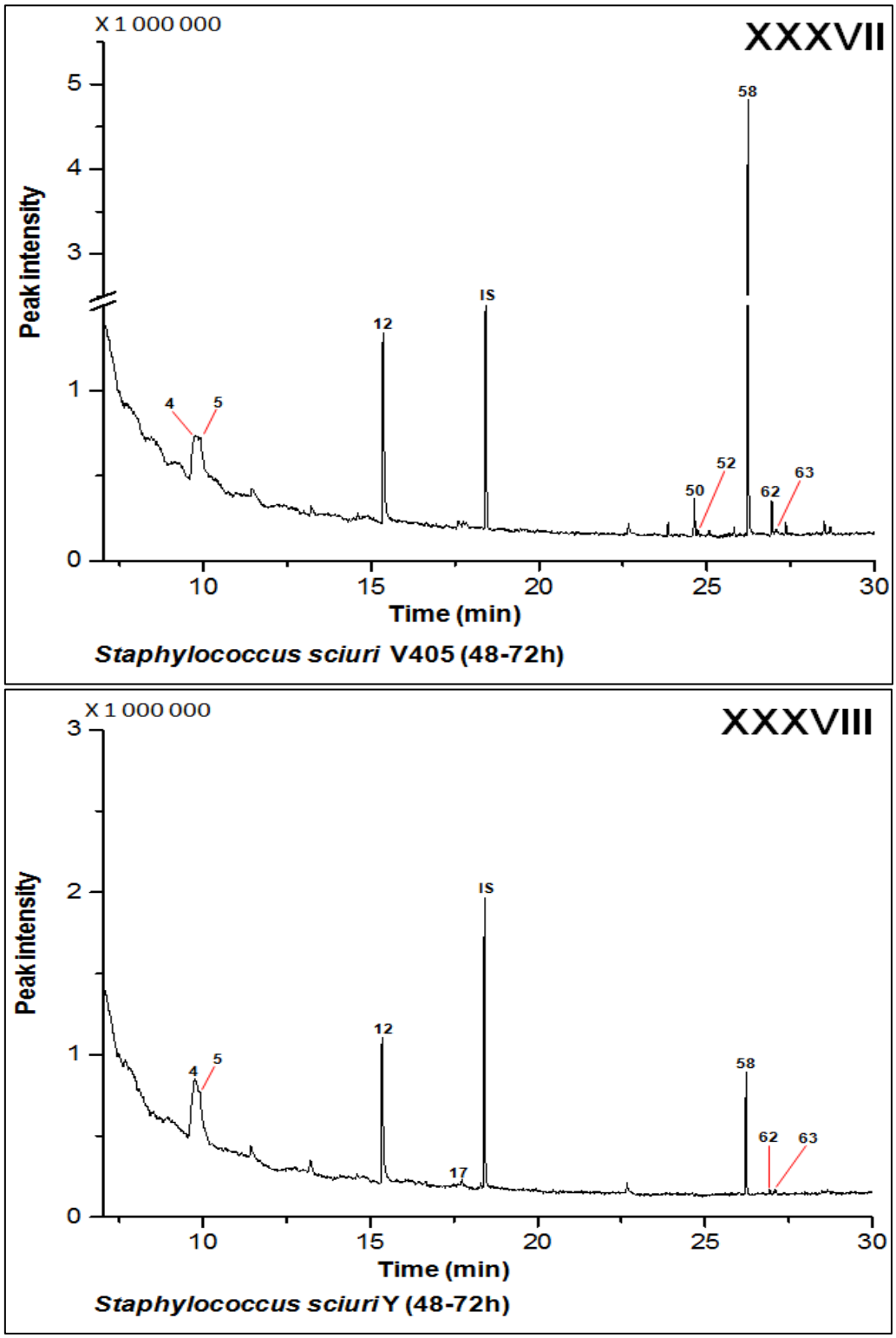


Figure S1: Chromatograms of various species and isolates of *Corynebacteriaceae* and *Staphylococcaceae*.

Bacteria were grown in BHI (brain heart infusion) medium at 30 °C. Headspace volatiles were collected at indicated time intervals and analyzed by GC/MS. Compounds are numbered

and summarized in Table S1. Chromatograms where most compounds of the total volatile profiles of each bacterium were present are depicted in I-II and IV-XXXVIII.

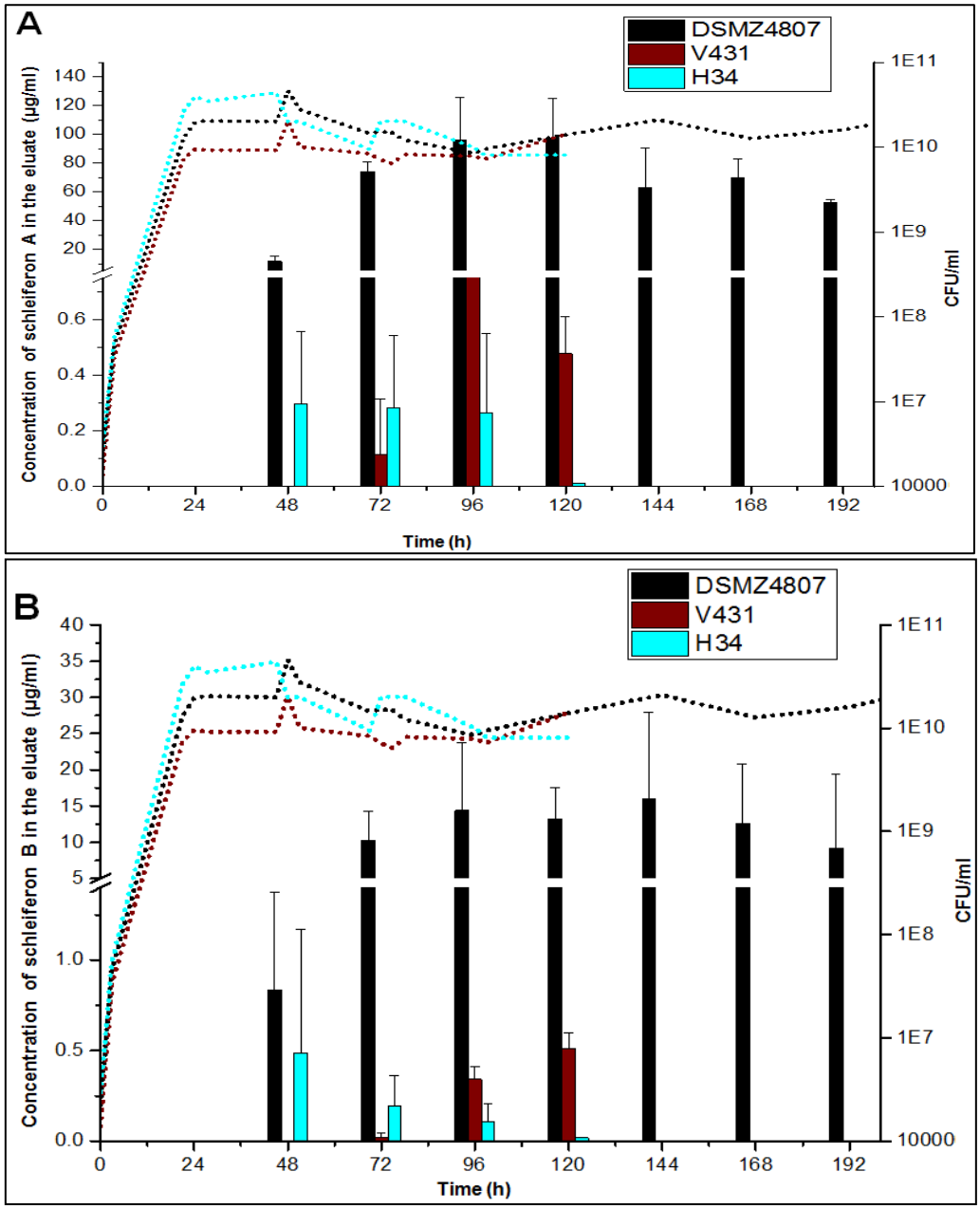
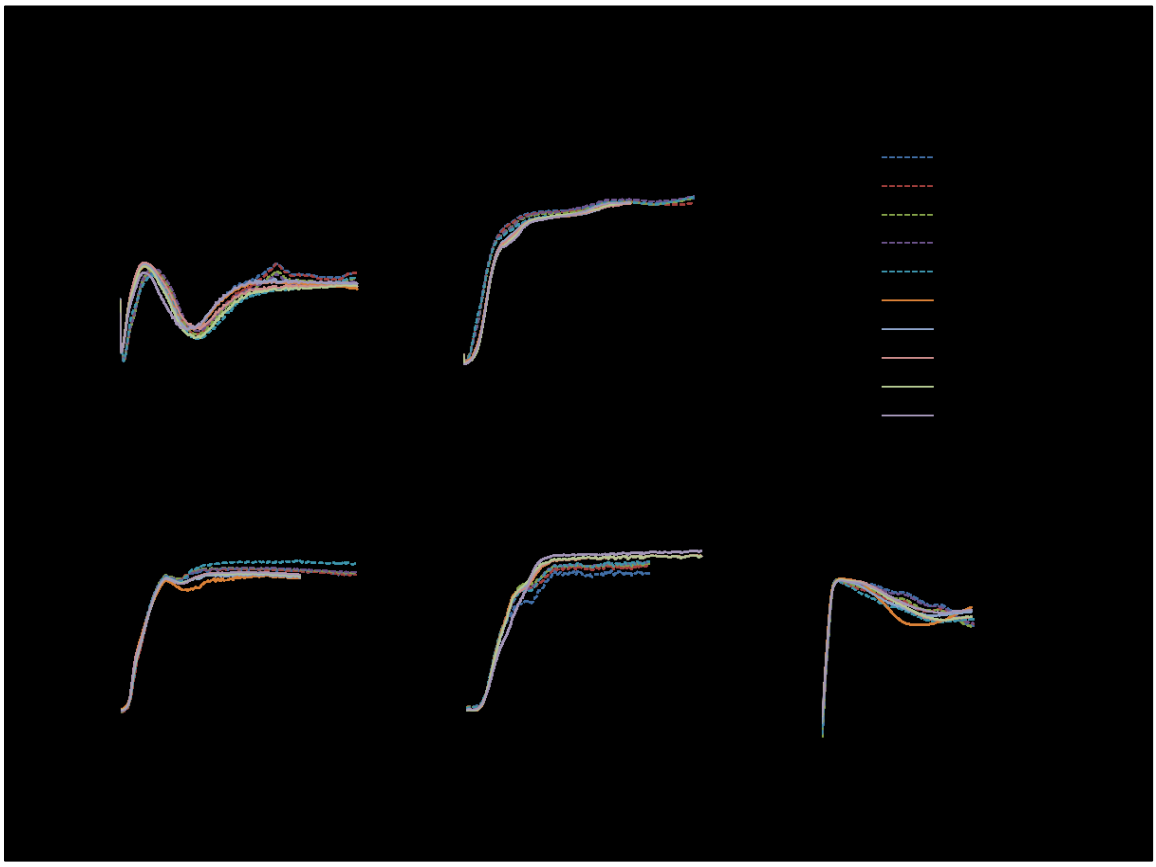
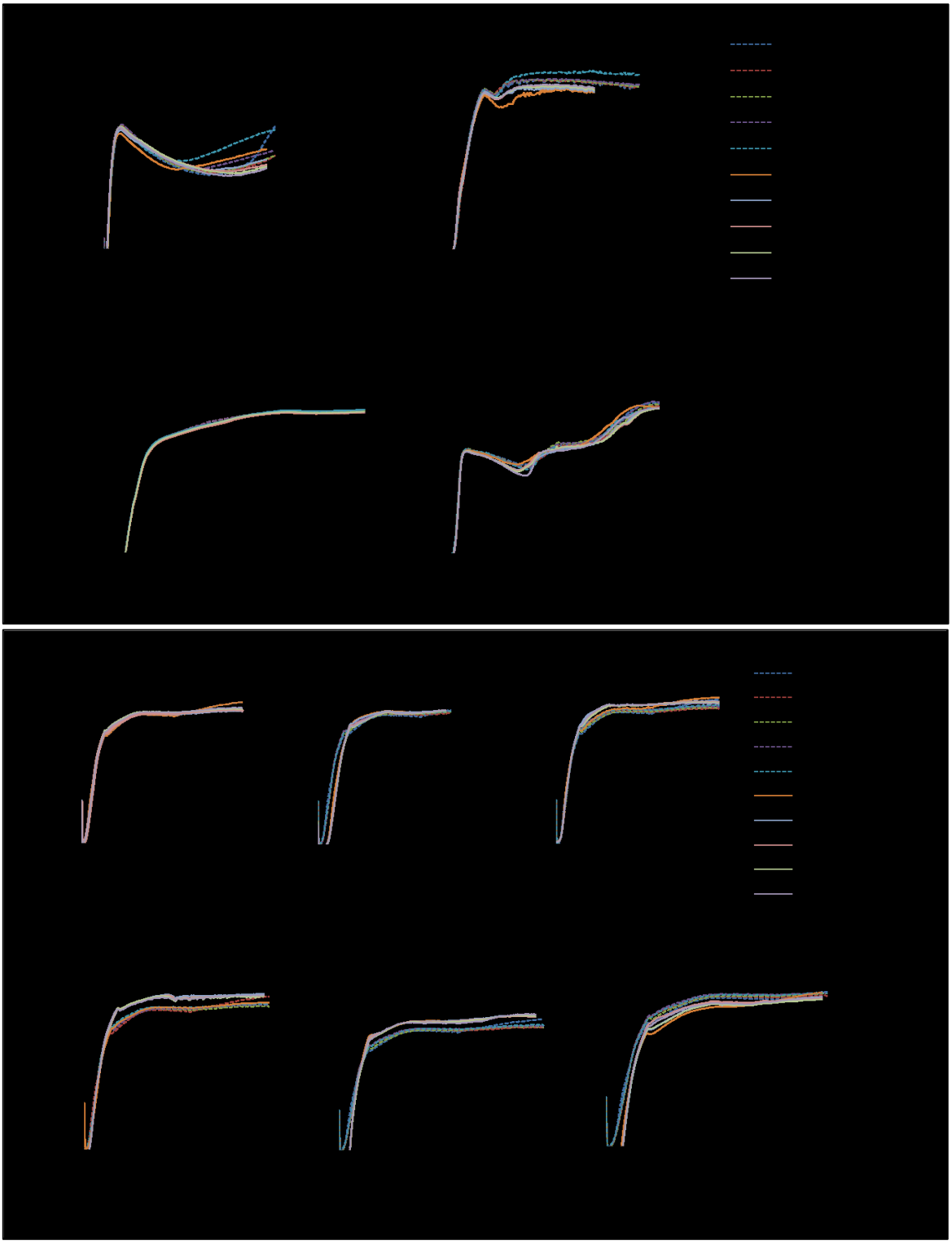


Figure S2: Production of schleiferon A (A) and schleiferon B (B) by three *Staphylococcus schleiferi* isolates.

The VOCs were collected at intervals of 24 h and analyzed by GC/MS. The total volume of the eluate was 310 µL (300 µL dichloromethane as solvent and 10 µL nonyl acetate (5 ng μL^{-1} final concentrations in the eluate) as the internal standard). The quantities of schleiferons A

and B produced were calculated based on the internal standard. Data are means of 3-4 independent experiments and bars indicate the mean standard deviation. The growth of the bacteria (100 mL bacterial culture) was monitored by determining the living cell numbers (CFU).





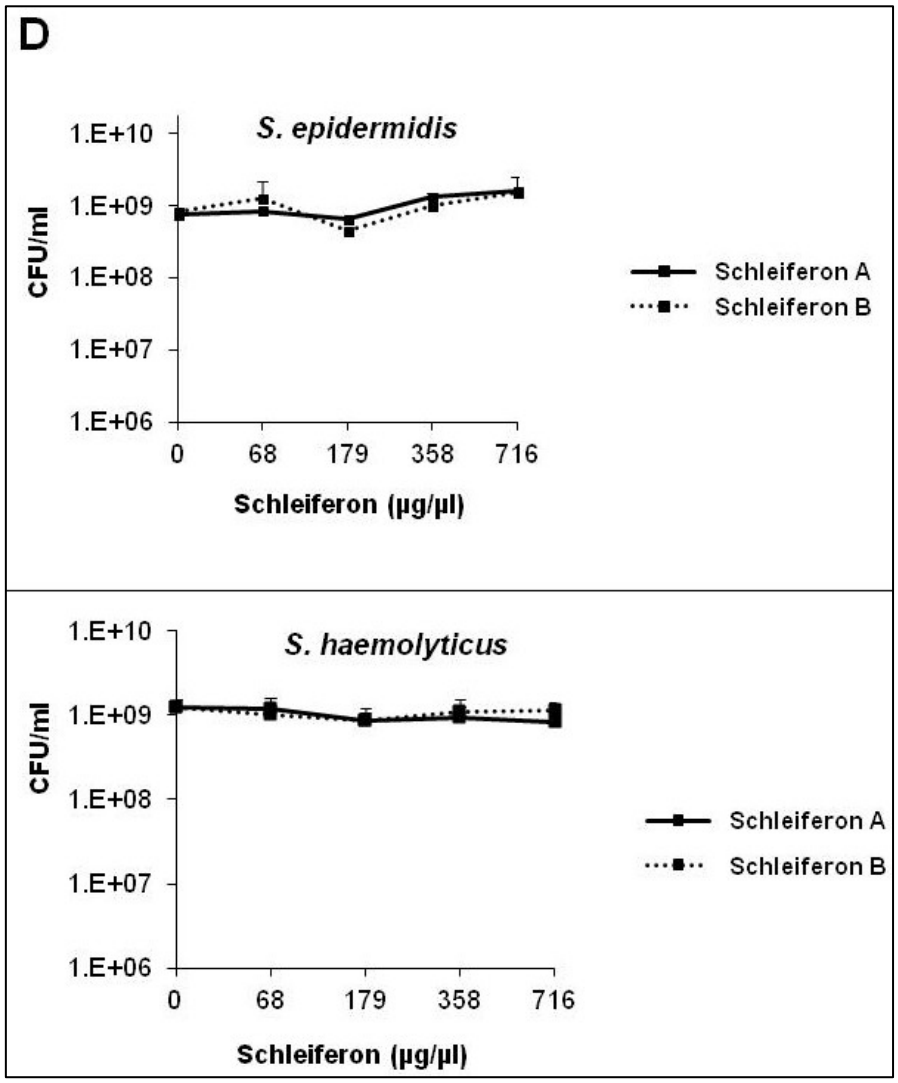


Figure S3: Effect of *Staphylococcus schleiferi* DSMZ 4807 volatiles and synthetic schleiferons A and B on different bacteria.

(A, B, C): Co-cultivation of *Staphylococcus schleiferi* DSMZ 4807 with different Gram-positive and Gram-negative bacteria.

Each test bacterium was co-cultivated with *S. schleiferi* (fresh) in 96-well microtiter plates at 30 °C. The growth of each Gram-positive **(A)** and Gram-negative **(B)** bacterium in dual culture with *S. schleiferi* was monitored by measuring cell density every 30 minutes at OD_{600} with a microtiter plate reader for 72 h or 96 h. Control: instead of *S. schleiferi* the medium (BHI) was added. The growth of *S. schleiferi* **(C)** in co-cultivation with test bacteria was also determined. Control: the culture medium was added instead of the test bacteria.

(D) Effect of synthetic schleiferons A and B on *Staphylococcus epidermidis* RP62A and *Staphylococcus haemolyticus* CCM 2729.

Bacteria were grown in BHI medium at 30 °C, in bipartite Petri dishes. A total of 4 mL of each bacterial culture (final OD 0.005) were transferred to one side of a bipartite Petri dish and 20 µL of various concentrations of schleiferon A or B solved in DMSO were added onto 6 mm Whatman filter paper disks, which were placed on the other side of the Petri dish. The plates were incubated for 20 h and viable cells (CFU) were determined. Data are the means of three independent experiments and bars indicate the mean standard deviation. When error bars are not visible, they were smaller than the symbols.

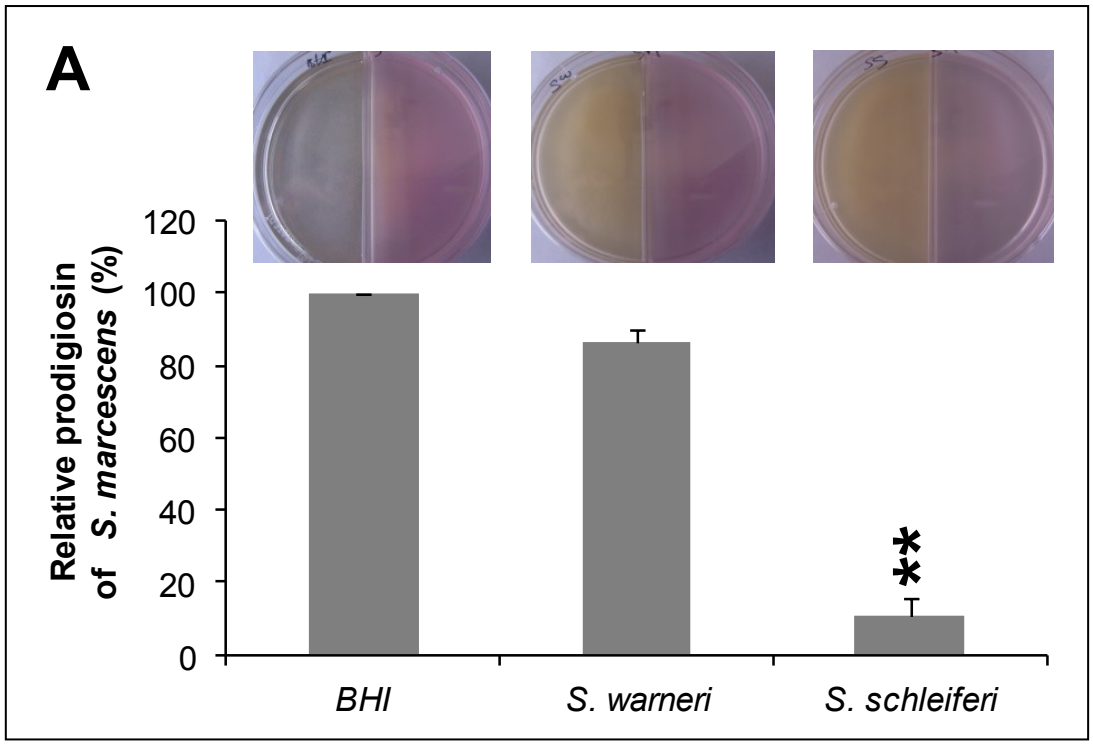


Figure S4A: Effects of *Staphylococcus schleiferi* and *S. warneri* volatiles on prodigiosin production by *Serratia marcescens*.

The concentration of the pigment was determined by measuring its absorption at OD_{534} and is expressed as a percentage of the level produced in the BHI control culture. ** $p < 0.01$.

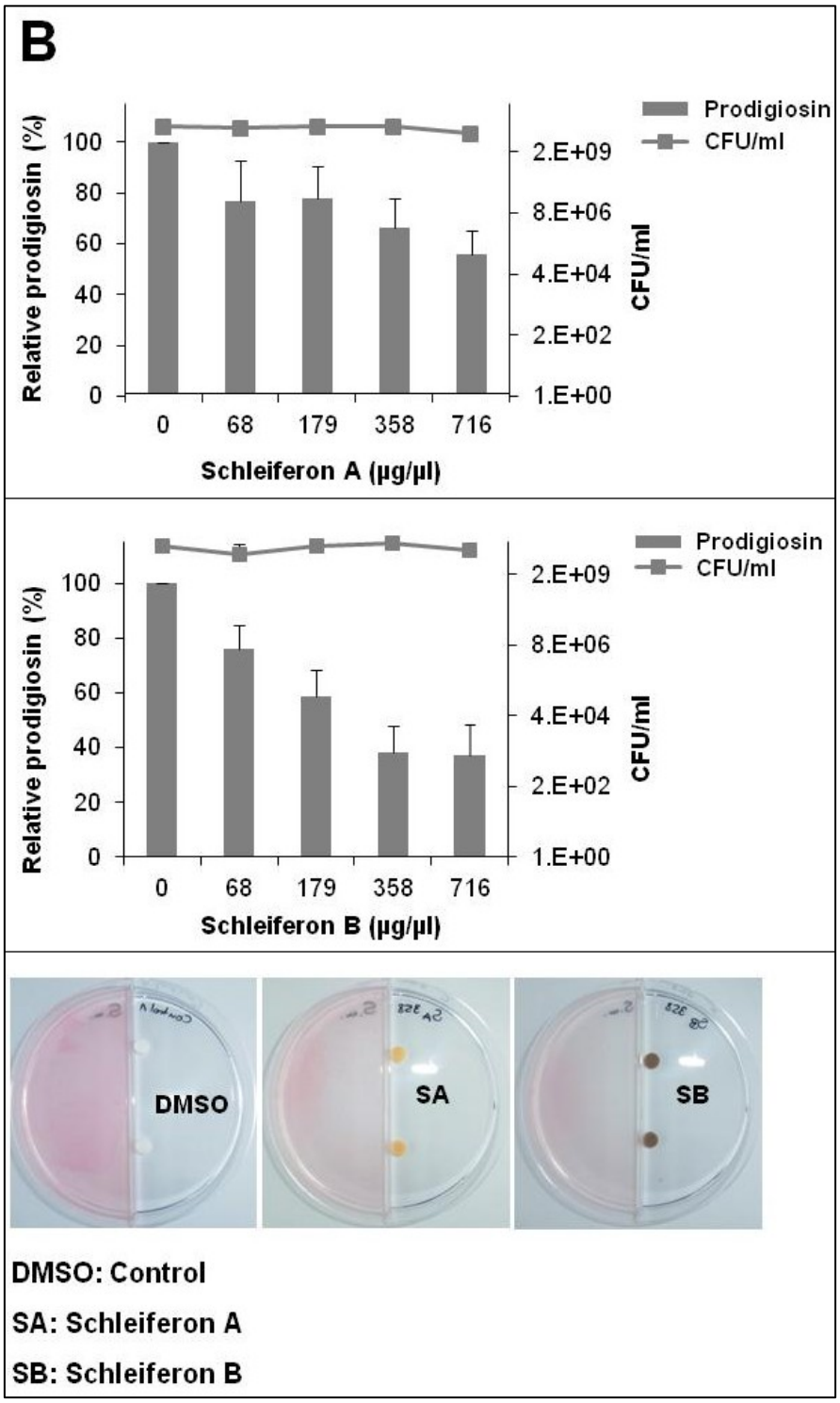


Figure S4B: Inhibition of prodigiosin production in *Serratia* by synthetic schleiferons A and B.

Bacteria were incubated with 20 µL increasing concentrations of schleiferon A or B. Bacterial growth was monitored by determining CFU, prodigiosin levels were determined by OD_{534} and

relative prodigiosin production was calculated as the ratio between prodigiosin and the CFU. Controls: bacteria were incubated with DMSO and their prodigiosin levels were set as 100% (=0.8 OD_{534}). Data are means of at least three independent experiments and bars indicate the mean standard deviation.

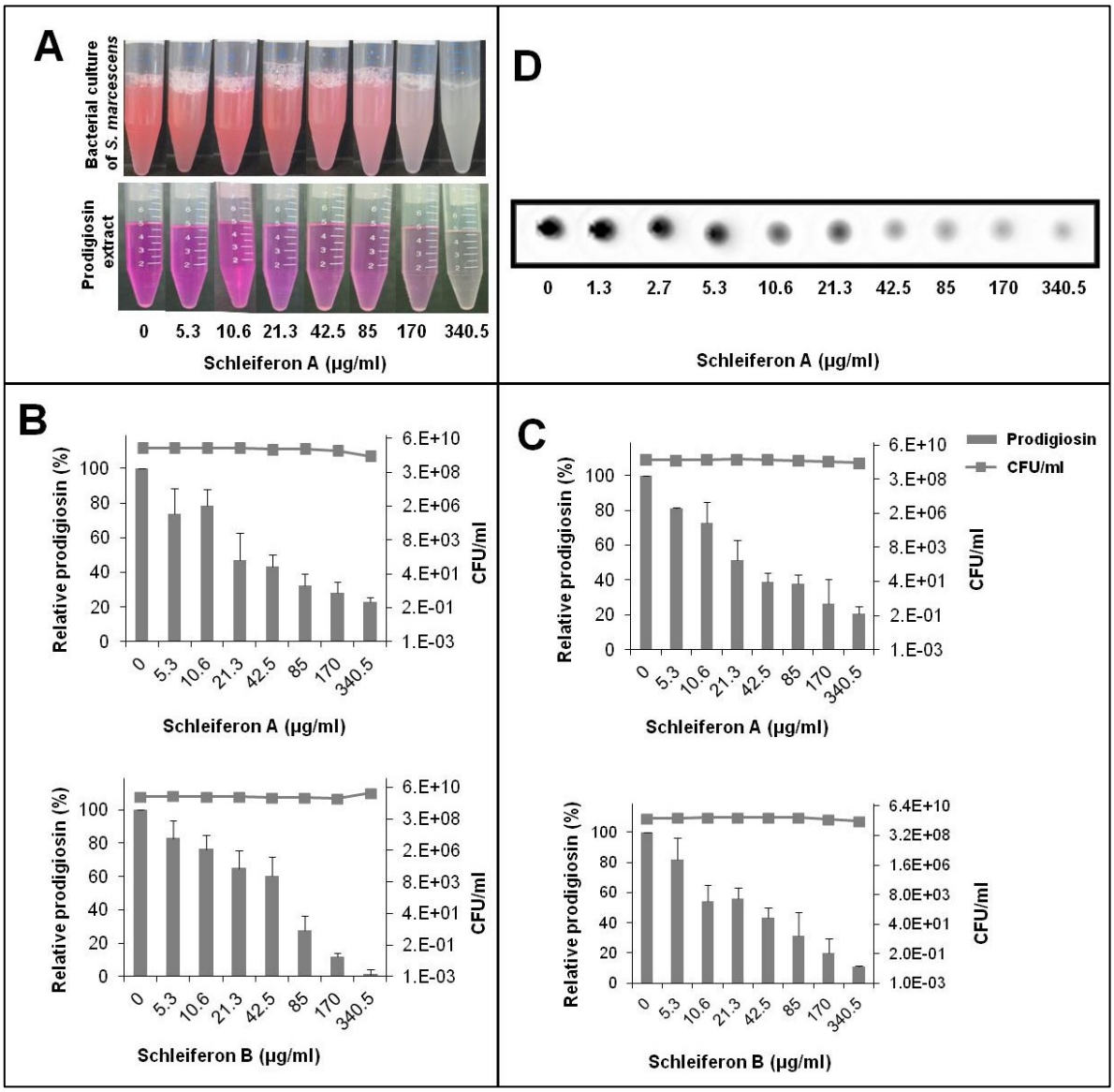


Figure S5: Inhibition of prodigiosin production in *Serratia marcescens* and *Serratia plymuthica* and bioluminescence of *Vibrio harveyi* by synthetic schleiferon A and B.

The bacteria were incubated with increasing concentrations of schleiferon A or B (final concentrations are shown). (**A**) The upper panel shows a *S. marcescens* V11649 culture after 20 h incubation at 30 °C, while the lower panel shows prodigiosin extracted from the bacterial

cells with acidified ethanol. Relative prodigiosin production (%) of *S. marcescens* V11649 (prodigiosin value of 100% = 2) (**B**), as well as *S. plymuthica* AS9 (100% = 0.98) (**C**), were calculated as the ratio between the prodigiosin content and living cell numbers (CFU mL^{-1}). Controls: the bacteria were incubated with DMSO and prodigiosin levels were set as 100%. (**D**) Spot tests showing relative levels of bioluminescence of *V. harveyi* DSMZ 6904 after 20 h incubation at 30 °C. Data are means of at least three independent experiments and bars indicate the mean standard deviation.

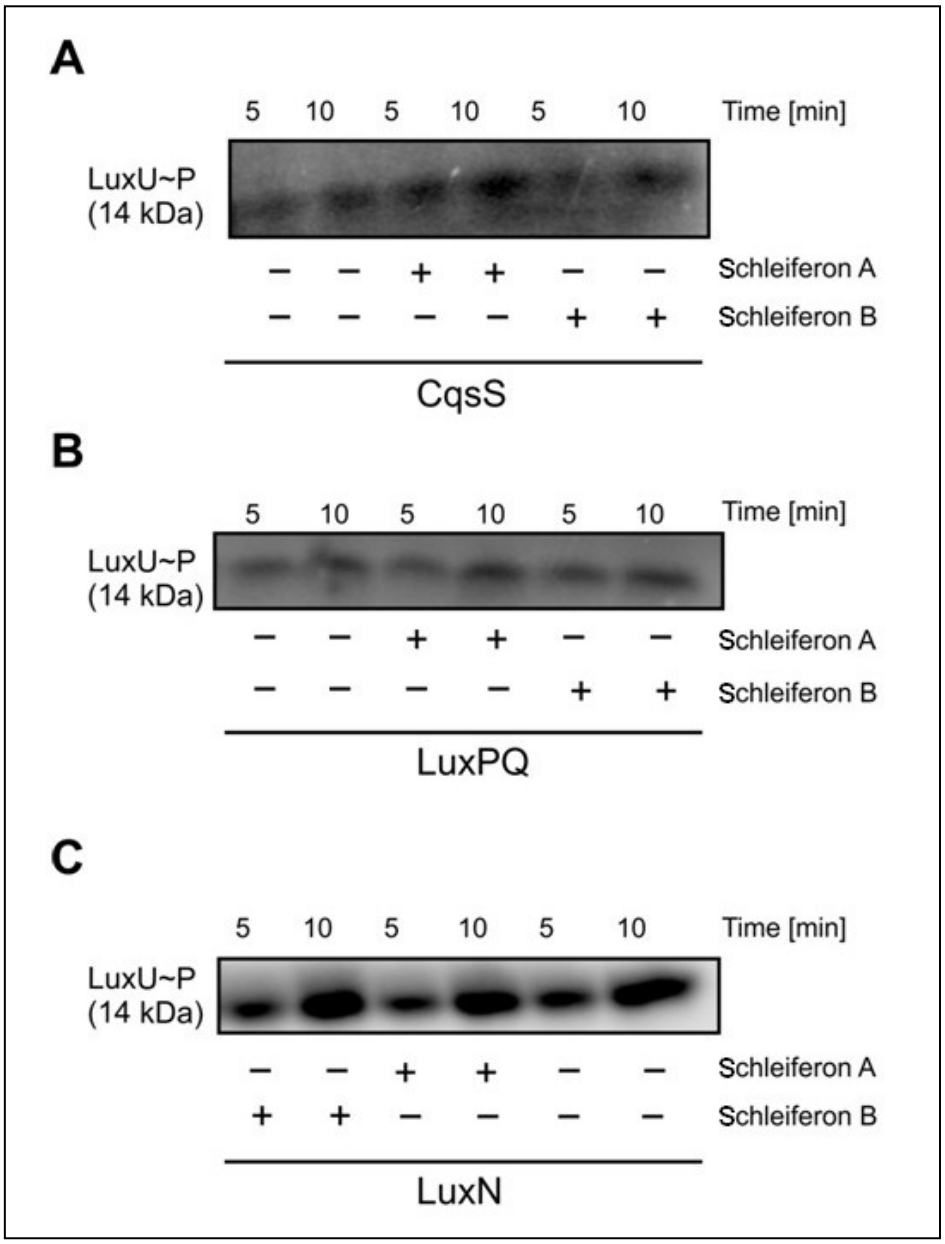


Figure S6: Effect of schleiferons A and B on CqsS (A), LuxPQ (B) and LuxN (C) mediated autophosphorylation and phosphotransfer to the HPr protein LuxU *in vitro*.
Inverted membrane vesicles containing the hybrid histidine kinases CqsS, LuxQ, and LuxN, respectively, were mixed with LuxU, and the reaction was started with [γ-^{32}P] Mg^{2+}ATP. When indicated, schleiferon A or B (340.5 µg mL^{-1} final concentration) or the corresponding volume of DMSO was added. For the LuxQ assay, LuxP had been incorporated into the vesicles. At the indicated times (5 and 10 min) the phosphorylation reaction was stopped, and proteins were separated by SDS-polyacrylamide gel electrophoresis followed by exposure of

the gels to a phosphoscreen. Autoradiographs of the LuxU parts of the gels are shown, and they are representative of three independent experiments.

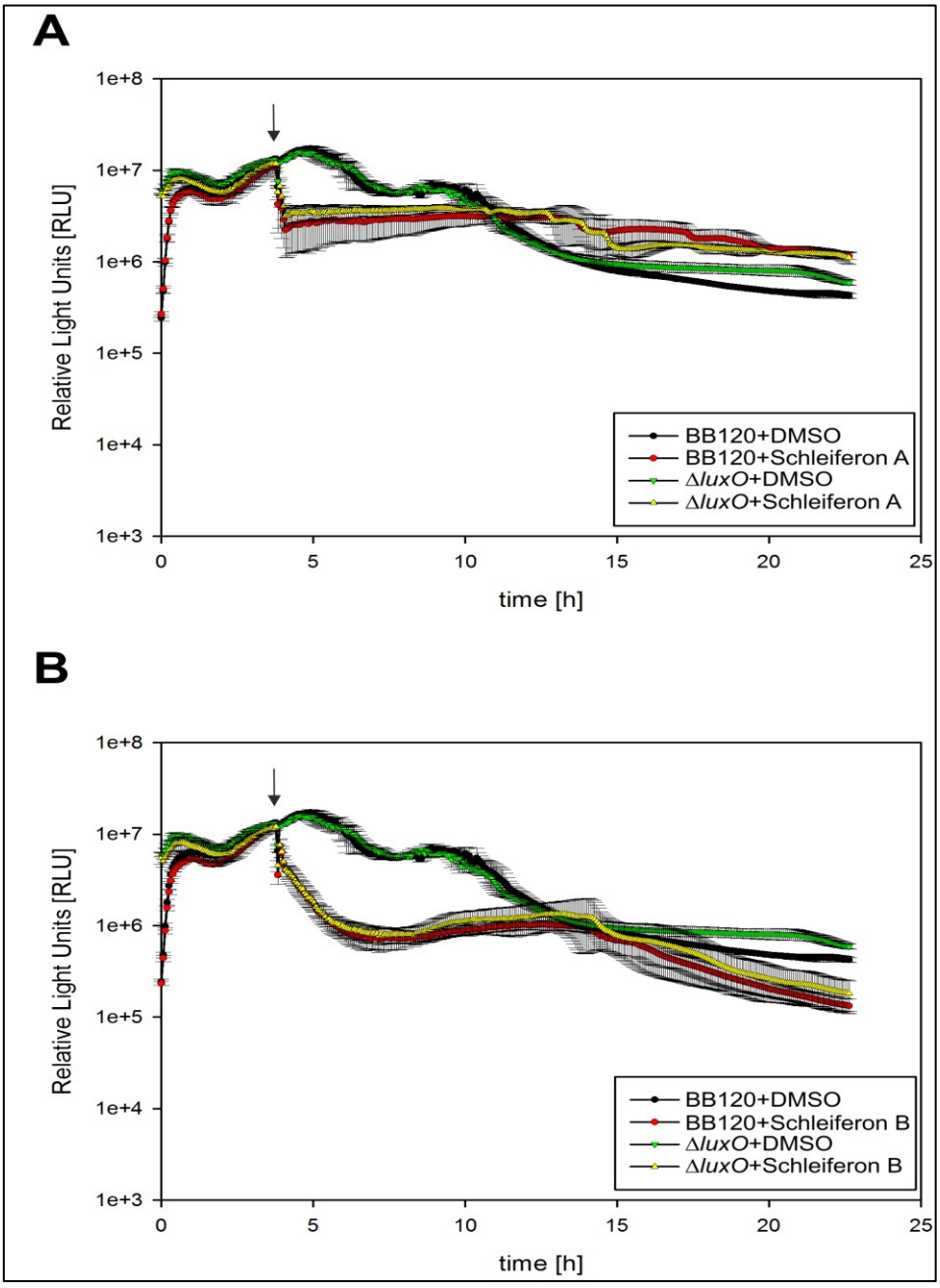


Figure S7: Inhibition of bioluminescence production of *V. harveyi* BB120 (wild type) and *ΔluxO* (constitutive QS-ON mutant) by schleiferons A (A) and B (B).

Cells from an overnight culture were inoculated in fresh AB-medium and grown aerobically at 30 °C in microtiter plates using a Tecan Infinite® F500 system. Bioluminescence and growth (OD_{600}) were recorded every 20 min. Schleiferons A **(A)** or B **(B)** were added to the

exponential growth phase at a final concentration of 340 µg mL^{-1} (indicated by the arrow). The corresponding volume of DMSO was added to the cells as a negative control. Error bars represent the standard deviation calculated from three independent replicates. RLU, relative light units in counts per second per milliliter per OD_{600}.

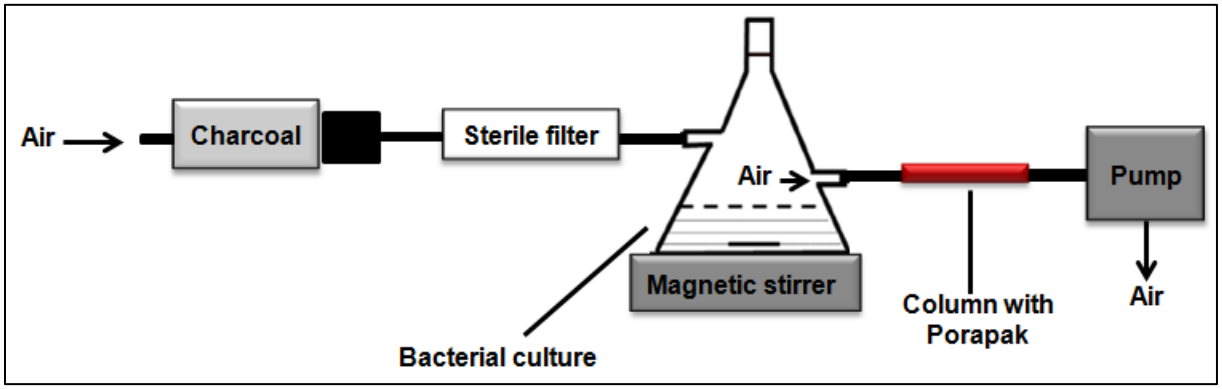


Figure S8: VOC collection system.

Charcoal-purified and sterile air entered the conical flask containing the bacterial culture. A trap filled with Porapak™ was connected to the outlet of the pump, which sucked the air and volatiles in the headspace of the bacterial culture into the trap.

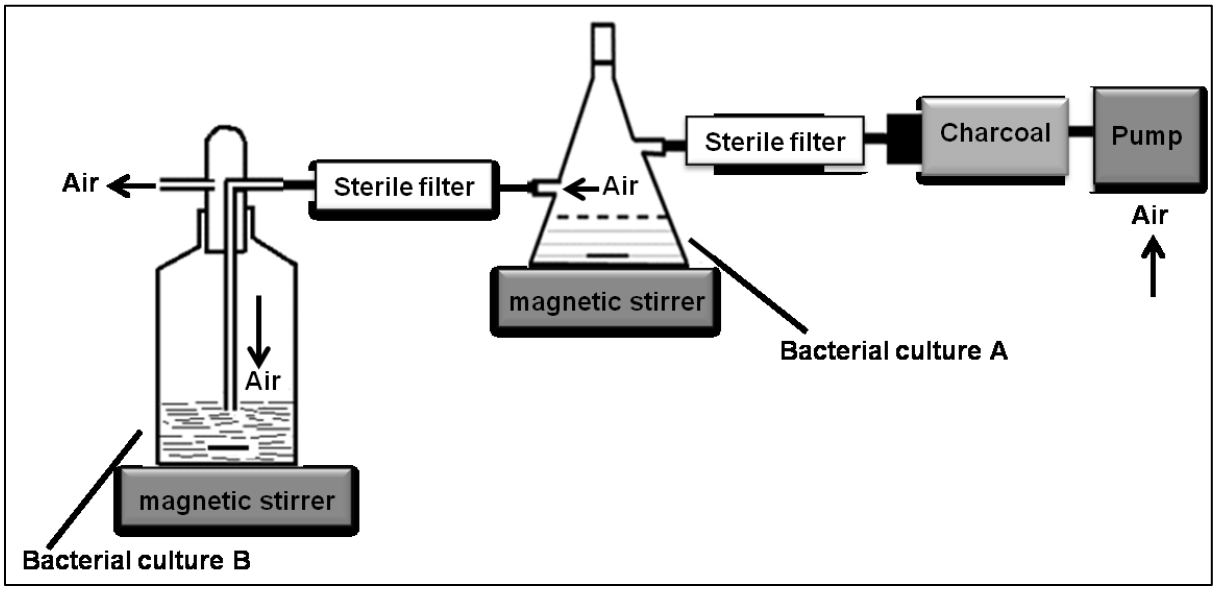


Figure S9: Dual culture system.

Charcoal-purified and sterile air was pumped through the inlet of flask A. The air enriched with the headspace volatiles of the bacterial cultures *S. schleiferi* or *S. warneri* (grown for 48 h or 96 h) entered the second flask B containing the bacterial cultures of *S. marcescens* or *V. harveyi*.

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4. Appendix

4.1. Additional results obtained which were so far not included in any publication

Volatile profile of skin bacteria

Table 1A: Volatiles of *Pseudomonas fluorescens* and *Micrococcus luteus*.

Compound			Bacteria	
numbers	**Compound names**	**RI**	***Pseudomonas fluorescens***	***Micrococcus luteus***
1	1-undecene	1064	*	
2	NI	1283	*	
3	NI	1370	*	
4	NI	944		*
5	NI	1108		*
6	NI	1128		*
7	NI	1134		*
8	NI	1225		*
9	NI	1323		*
10	NI	1330		*
11	NI	1338		*
12	NI	1421		*
13	11-methyl-2-tridecanone	1520		*
14	10-methyl-2-tridecanone	1528		*
15	NI	1535		*
16	NI	1619		*
17	2-pentadecanone	1653		*
18	NI	1725		*
19	NI	1843		*

The bacteria were grown on BHI medium for 96 h at 30 ° C. Volatiles of the headspace of bacterial cultures were analysed in 24 h intervals by GC-MS. Compounds were numbered as shown in the chromatograms (figure 1A and 2A) were identified by comparing their retention index and mass spectra with the NIST 107 mass spectral library (version 1998), NIST Chemistry WebBook and with authentic standards. NI: not identified.

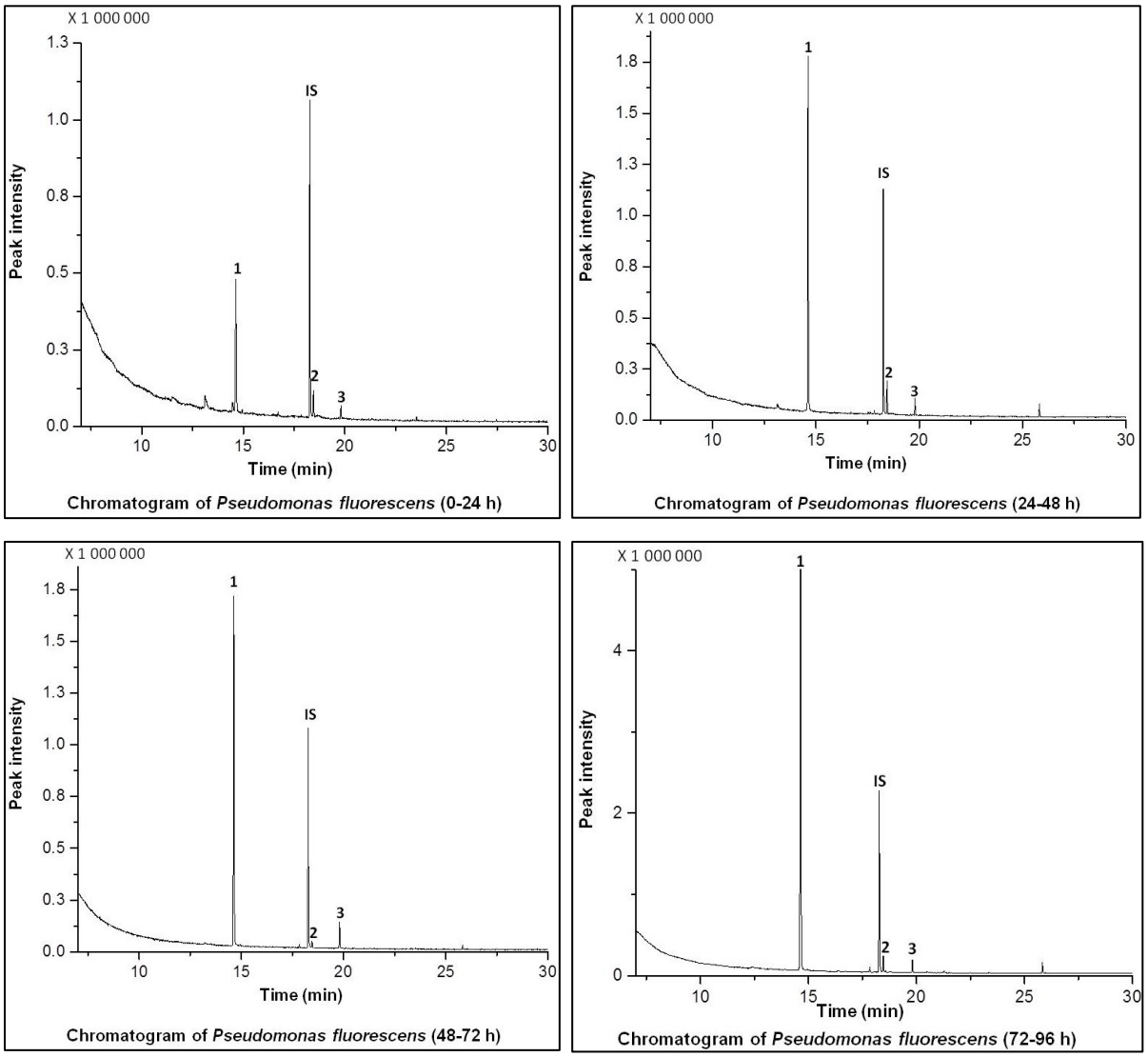

Figure 1A: Chromatograms of *Pseudomonas fluorescens* V1411.

Bacteria were grown on BHI (brain heart infusion) medium at 30° C. Headspace volatiles were collected at different time intervals (each 24 h) and analysed by GC-MS. Compounds were numbered and summarized in Table 1A.

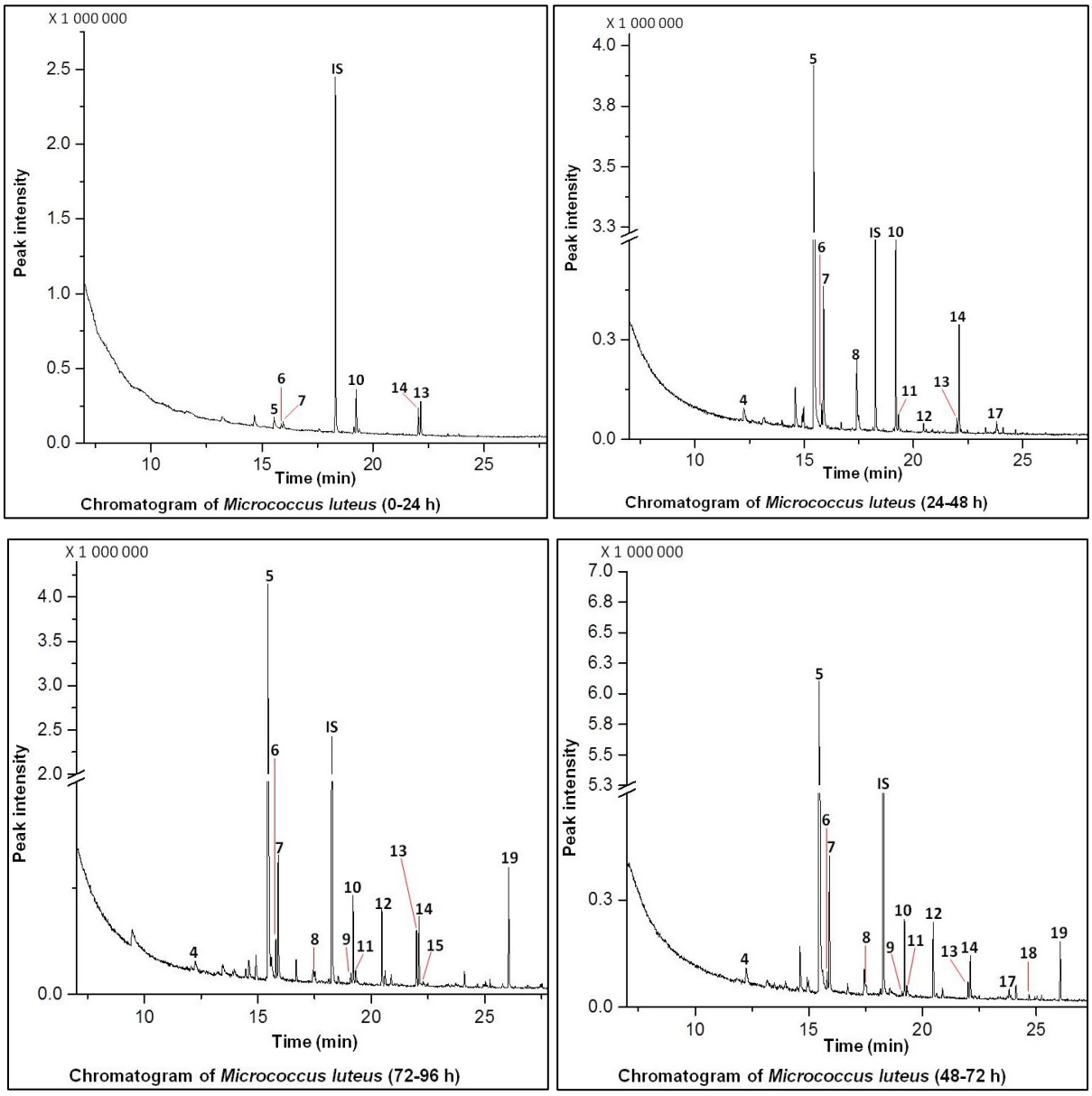

Figure 2A: Chromatograms of *Micrococcus luteus* V515.

Bacteria were grown on BHI (brain heart infusion) medium at 30° C. Headspace volatiles were collected at different time intervals (each 24 h) and analysed by GC-MS. Compounds were numbered and summarized in Table 1A.

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4.3. Complete list of publications

1- **Lemfack, M.C.**, Ravella, S.R., Lorenz, N., Kai, M., Jung, K., Schulz, S., Piechulla, B. (2016) Novel volatiles of the skin-borne bacteria inhibit the growth of Gram-positive bacteria and affect quorum-sensing controlled phenotypes of Gram-negative bacteria. Syst. Appl. Microbiol. In revision.

2- Piechulla, B., **Lemfack, M.C**. (2016) Microbial volatiles and their biotechnological applications. In: Arimura, G., Maffei, M (eds), Plant Specialized Metabolism: Genomics, biochemistry and biological function. CRC Press 10, 239 - 256.

3- Schenkel, D., **Lemfack, M. C.**, Piechulla, B., Splivallo, R. (2015) A meta-analysis approach for assessing the diversity and specificity of belowground root and microbial volatiles. Frontiers in Plant Science 6, 707.

4- **Lemfack M.C.**, Piechulla B. (2014) mVOC -online-Datenbank mikrobieller Geruch-stoffe. Naturwissenschaftliche Rundschau 4, 211.

5- **Lemfack, M.C.**, Nickel, J., Dunkel, M., Preissner, R., Piechulla, B. (2013) mVOC: a database of microbial volatiles. Nucl. Acids Res. 42, 744-748.

6- Telefo, P.B., **Lemfack, M.C.**, Bayala, B., Lienou, L.L., Goka, C.S., Yemele, M.D., Mouokeu, C., Tagne, S.R., Moundipa, F.P. (2012) Enquête ethnopharmacologique des plantes utilisées dans le traitement de l'infertilité féminine dans les localités de Fossong-Wentcheng et Foto, Cameroun. Phytothérapie, 10, 25-34.

7- Telefo, P.B., Lienou, L.L., Yemele, M.D., **Lemfack, M.C.**, Mouokeu, C., Goka, C.S., Tagne, S.R., Moundipa, F.P. (2011) Ethnopharmacological survey of plants used for the treatment of female infertility in Baham, Cameroon. Journal of Ethnopharmacology 136, 178-187.

8- Lienou, L.L., Telefo, P.B., Bayala, B., Yemele, D.M., **Lemfack, M.C.**, Mouokeu, C., Goka, S.C., Tagne, R.S.,Moundipa, P.F. (2010) Effect of ethanolic extract of Senecio biafrae on puberty onset and fertility in immature female rat. Cameroon Journal of Experimental Biology 6.

4.4. Participation in conferences

Poster presentation

Lemfack, M.C., Nickel, J., Dunkel, M., Preissner, R., Piechulla, B. Identification of new bacterial scent compounds and introduction of the first database of microbial volatiles "mVOC". 4th Joint meeting of the Association for General and Applied Microbiology (VAAM) and the German Society for Hygiene and Microbiology (DGHM), Dresden 05.-08.10.2014 (**poster price**).

Oral presentations

Lemfack, M.C., Kai, M., Piechulla, B. Volatiles of symbiotic bacteria of the human skin microbiome. Annual meeting of the Association for General and Applied Microbiology (VAAM), Marburg 01. - 04. 04. 2015.

Lemfack, M.C., Piechulla, B. *Staphylococcus schleiferi* DSMZ 4807 volatiles inhibit quorum sensing controlled phenotypes in Gram-negative bacteria. Annual meeting of the Association for General and Applied Microbiology (VAAM), Jena 13.-16. 04. 2016.

Lemfack, M.C., Piechulla, B. Volatiles of the human skin microbiome act as quorum quencher. Annual conference of the International Society of Chemical Ecology (ISCE), Iguazu (Brazil) 04.-08.07.2016 (**best oral presentation price**).

4.5. Declaration of the doctoral candidate

According to § 4 (1) letters g and h of the doctoral degree regulations of the Faculty of Mathematics and Natural Sciences of the University of Rostock

Lemfack Marie Chantal

Ziolkowski Str. 8A, 18059 Rostock

I intend to elaborate a dissertation on the topic
Development of the first database of microbial volatile organic compounds and Analysis of the skin bacterial volatiles and their effect in bacteria-bacteria interactions
at the Faculty of Mathematics and Natural Sciences at the University of Rostock.

My supervisor is Ms Prof. Dr. Birgit Piechulla.

I, herewith, declare the following:

1. The opportunity for this PhD project was not communicated to me commercially. In particular I have not engaged any organisation that for money seeks supervisors for the drawing up of dissertations or that performs entirely or partially on my behalf the duties incumbent upon me regarding the examinations.

2. I hereby declare under oath that I have completed the work submitted here independently and have composed it without outside assistance. Furthermore, I have not used anything other than the resources and sources stated and where I have taken sections from these works in terms of content or text, I have identified this appropriately.

Rostock..... this ....