

Serratia- and Bacillus-produced Volatiles and their fungistatic properties on Rhizopus stolonifer

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Abstract

Serratia marcescens lives a lifestyle of diversity and ubiquity. Found nearly everywhere this bacteria is capable of living as a saprobe in soil and water, but also living in community with plants and other rhizobacteria, and in addition as a pathogen to plants, animals and other microbes. Besides being a beautiful example of life's ability to adapt to survive, Serratia also provides insight to one interesting aspect of microbiology. How do bacteria and fungi communicate? We have identified multiple strains of Serratia from unique niches and compared their ability to inhibit the growth of common soil inhabitants known to interact with plants, Mucoromycota.

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Importance:

This research shows bacteria that live in the soil (*Serratia marcescens*, *Serratia proteamaculans*, and *Bacillus subtilis*) are able to produce compounds (small molecules) that are then volatilized (evaporate into the air) that can travel distances and slow or prevent fungi from growing. A common fungi known as Black Bread Mold, aka *Rhizopus stolonifer*, can often be found in the soil or rotting strawberries. We have shown in the laboratory that bacteria can stop or slow the growth of this fungi when they share the same air. Each bacteria had many compounds that were volatiles and some were unique to the bacteria that made them. We used purified chemical compounds we found in the bacterial gas and tested them one at a time to see if they work as antifungals. We found that all the tested bacteria produced several compounds that are fungistatic (stops fungal growth). Future work may include looking at the volatiles produced by the bacteria living in the soil or other environments where *Rhizopus* may come in contact.

Introduction:

Interactions between Fungi and Bacteria span mutualistic, such as bacteria using fungal hyphae as highways [1], to antagonistic as seen in the famous penicillin discovery [2]. Improvements in imaging and sequencing approaches have aided in illuminating the niches where fungi and bacteria communities coexist, communicate and cooperate. Such as metagenomics in lichen symbioses of cyanobacteria and fungi [3], and what organisms are competition within the topsoil [4], or coexisting in extreme environments [5]. Tools for microbiomics have enabled studies of how fungal and bacterial. For example, *Aspergillus nidulans* was found to activate secondary metabolites after physical contact with *Streptococcus*, these metabolites are similar to lecanoric acid found in lichen symbiosis but also functions as a defense compound inhibiting ATP production [6]. Bacteria may help fungi to be better pathogens by creating toxins like Rhizoxin [7]. Bacterial-fungal communities are unique and diverse leading to discoveries from studying these relationships include finding that early lineages of fungi harbor ecto- and endohyphal bacteria and their role in asexual reproduction, nitrogen, and carbon utilization [8–14][6,7,15][8–14], mechanisms and applications of bacterial-fungal interactions [16], and microbial volatile bouquets [17]. Bacteria utilize fungal hyphae for high-speed travel, they can eat fungi, and they can consume the metabolites. [1,18–20]. These relationships have yielded many benefits including furthering our understanding of nutrient cycling in ecosystems, fermented foods and beverages, and antibiotics [21].

Microbial genomes encode genes for many antifungals and antibiotics [22]. Some studies have highlighted antagonism, table 1. Such as work has brought about the approval of *Serratia plymuthica* as a biocontrol (Rhizostar) that reduces *Verticillium* wilt by 18.5% and *Phytophthora cactorum* root rot by 33.4% in strawberry [23]. A conserved response to bacteria was demonstrated when antagonistic *Lysobacter* enzymes was shown to repress fungal response genes with effectors. Another repressor produced by *Serratia marcescens* (Smar), inhibits the growth of *Candida albicans*, [24]. Recently, *Fusarium fujikuroi* and *Botrytis cinerea* were shown to protect their survival spores after exposure to the bacterial secondary metabolite, ralstonin, from *Ralstonia solanacearum* [25]. To add to the complexity, fungal-bacterial interactions occur among communities of organisms where detection and specific response to organisms from plants to animals may be required [16,21].

| Bacteria | Fungi/Oomycete | Reference |
|-------------------------|--|---------------|
| Bacillus megaterium | Aspergillus flavus | [25] |
| Bacillus subtilis | Aspergillus niger, Alternaria alternata, Botrytis cinerea, Colletotrichum gloeosporioides, Fonsecaea pedrosoi, Fusarium oxysporum, Monilinia fructicola, and Penicillium expansum | [26–29] |
| Serratia proteamaculans | Helminthosporium sativum, Rhizoctonia solani, and Sclerotinia sclerotiorum | [30,31] |
| Serratia plymuthica | Batrachochytrium dendrobatidis, Batrachochytrium salamandrivorans, Phytophthora cactorum, Rhizoctonia solani, Verticillium dahliae | [22,31–34] |
| Serratia marcescens | Batrachochytrium dendrobatidis, Batrachochytrium salamandrivorans, Candida albicans, Diaphorina citri, Didymella applanata, Lasiodiplodia theobromae, Pythium ultimum, Phytophthora parasitica, Phytophthora cinnamomi, Phytophthora citrophora | [23,32,35–39] |
| NA | Saccharomyces cerevisiae, Curvularia eragrostidis, Pestalotiopsis theae, Colletotrichum camelliae, Lasiodiplodia theobromae, Rhizoctonia solani | NA |

Although fungi can respond to direct contact with bacterial microbe-associated molecular patterns [32], strangely, fungi seem to lack common eukaryotic receptors for bacterial recognition [33]. The lack of receptors may be a result of an effective fungal offense e.g. producing antibiotics, sequestering iron, making their general vicinity inhospitable to bacterial invasion through decreasing pH [32,34]. Due to the fact that microbes may

be detrimental to each other, perceiving infochemicals from neighboring competitors or acting on each other from a distance is an important and understudied aspect and additionally, what are the volatiles produced by microbes [35,36]. Bacteria produce volatiles which can diffuse in the soil, and have been shown to be useful communication signals [22,37–39]. The impact of some volatiles can be harmful to organisms, or may serve as infochemicals which fungi may respond to by avoidance, morphology changes, or arresting growth [34]. Some bacteria like *Serratia* closely interact with plant roots, and form physical barriers barring potential pathogens, in addition, they can provide nutrients and growth hormones like indole-3-acetic acid to the plant [40–43]. *Serratia* is widely considered a rhizobacteria but how does it interact with the fungal populations nearby? The fungal growth inhibition by volatiles has been shown in a few systems such as between plants and their pathogens [44] as well as bacteria and fungi (*Ralstonia* and *Fusarium* and *Botrytis*) [25]. Fungal-bacterial communication has been neglected with the lack of tools to analyze the interactions, however, with transcriptomics, genomics, and metabolomics increasing in popularity and decreasing in cost more is being done. Rhizoctonia in direct contact with *Serratia plymuthica* had transcriptional responses that lead to hyphal morphology changes, defense, and toxin production [34]. The interest in indirect contact, volatiles, and their functions and applications are becoming more popular as well. The chemical diversity of volatiles produced by bacteria is broad with some having antifungal properties [45]. Because volatile profiles differ from bacteria to bacteria a resource called mVOC 2.0 was created showing volatile signatures of many bacteria [46]. A few key volatiles can be a sufficient signature to identify a microbe. In fact, plants identify many volatiles produced by bacteria as a way of communication [47]. Volatiles pass through soil through diffusion and advection [48] which makes volatile communication between microbes effective, analogous to quorum sensing. *Smar* is a rhizobacterium and inhabits soils among many environments [49]. *Serratia* is an effective biocontrol agent due to a number of antimicrobials and enzymes that it secretes. Prodigiosin, a red-pigmented tripyrole compound that has been shown to inhibit mycelial growth and it is secreted into the environment, in fact, traces of prodigiosin have been found in soil of tea plants [26]. *Serratia* can produce and secrete enzymes as well such as chitinases that digest fungal cell walls [24,50–52]. *Smar* has been shown to produce the potent anti-oomycete compound Oocydin which [53]. In addition to this arsenal of products, *Serratia* volatiles have been identified and shown to affect growth, antibiotic production and gene expression of neighboring bacteria [22,54]. Due to the metabolic cheapness and high vapor pressure, volatiles easily move around in the soil [36]. Most are thought to be waste products of biosynthetic pathways, but some unique volatiles such as γ -butyrolactones can inhibit a wide range of bacteria [55]. Other bacteria have been shown to produce volatiles that can inhibit spore germination, *Streptomyces* inhibits *Cladosporium* [56] and *Ralstonia* produces ralstonins that inhibit germination of *Aspergillus flavus* [57]. Even fungi can produce volatiles to affect other fungi. Yeast volatiles reduced the ability of *Aspergillus* to resist insect damage [58], showing that while volatiles may not be fungicidal they can lead to fungal death indirectly. One of the volatiles produced by *Serratia* is 2-undecanone [59], a non-hazardous volatile [59–61] that has fungistatic properties [62,63], efficacy as a mosquito repellent [64], and is used as a fragrance for its minty properties [65]. Undecanoic acid elicits a response in the fungus *Trichophyton rubrum* leading to up-regulation of transcripts involved in reduction of fatty acid and metabolize 2-undecanone, oxidative stress, and cell wall integrity loss [66].

Materials and Methods:

Strains: *Serratia marcescens* (*Smar*) - Lab strain ADJS-2C_Red [67]), *Serratia proteamaculans* (*Spro*) - Lab strain BW106 [1], *Bacillus subtilis* (*Bsub*) - Strain E9 [68], *Basidiobolus ranarum* (Lab strain: AG-B5), *Mucor circinelloides* (CBS 277.49), *Rhizopus stolonifer* (NRRL 66455), *Alternaria infectoria* (Lab strain DB1-7), and *Neurospora crassa* (FGSC 4289) Donut Plates: Luria-Bertani (LB) (10g Peptone, 10g NaCl, 5g Yeast Extract, 7.5g Agar, and 500mL Water) was pipetted into the outer ring of a 100mm petri dish with a 60mm petri dish lid placed inside. Then MEYE or Vogel’s Media was added to the 60mm petri dish lid. 1mL of S.m. was inoculated onto the outer LB ring and allowed to grow for 48 hours at 25 C. Then mycelial plugs or 1000 spores/conidia were added to the media in the central 60mm petri dish lid. Measurements were taken 48 hours after fungi were inoculated. *Bsub* was previously shown to be effective at inhibiting *Fusarium* [68] and was used as a positive control GC-MS: One colony was picked and grown overnight in liquid LB media, then the culture was inoculated onto three plastic Petri dishes and allowed to grow for 48

hours at 28°C, then volatiles were collected for 6 hours at 25°C. To serve as positive controls Bsub, Strain E9 was used. To serve as negative controls volatiles from blank Petri dishes and Petri dishes with LB media were collected. 1mL of S.m. grown overnight was added to a 60mm petri dish, headspace volatile collection was taken at 48 hours for 6 hours at 25°C at .5L/min with air cleaned by a charcoal purification filter pushed into to sample collection apparatus (350 mL Mason Ball jar). Jar lids were fitted with a Teflon liner and two brass bulkhead unions (pipe thread .64 cm Swagelok, San Diego Valve and fitting Co.) with output into a volatile trap. The volatile trap consisted of ¼ inch glass tube filled with 40 mg HayeSepQ beads 80-100 mesh size. Volatiles were eluted with 150 ul Dichloromethane spiked with 4ng/ul nonyl-acetate, 2 ng/ul octane and was stored at -80°C until GC:MS analysis. For GC coupled Mass spect carrier was helium, over ramp was 32 minute 40°C to 280°C. DB Wax column, TG-5MS Thermo fisher. Spectra were analyzed with Chromeleon 7 software and compounds were identified based on retention times and RSI (Reverse Match Factor) from the NIST library index. Volatile Analysis: Volatile amounts were calculated using area under the curve of a spike control of known mass. The volatiles with a prediction of 85% RSI or higher were kept. Unique volatiles were removed, those only produced by one sample or two samples. The PCA plots and NMDS were produced in R. NMDS was produced with the function metaMDS from the vegan package with the code on Github (https://github.com/Derreckadam/NMDS_Volatiles/tree/master). MetaboAnalyst 4.0 was used to further analyze samples. Samples were scaled, then ANOVA (w/ Tukey's Post Hoc) identified significantly different volatiles produced between the strains, and the clustering and sample distance was calculated in euclidean space by the Ward.D method. Random forest plot show separation of Bsub from the Serratia strains after just a few generations, but the convergence of the Serratia samples after 200 generations indicating that the two. Microscopy: 1000 spores from Rstol were plated onto 8 mL MEYE media with agar [69] in a 60 mm petri dish and allowed to grow for 12 hours at 25°C. The hyphae were then examined under an inverted compound microscope at 200x. In 2-undecanone treated samples, 1 mg of 2-undecanone was added near the edge of the hyphae on a piece of filter paper and exposed for 1 hour before imaging. Pure Volatile Serial Dilutions: 2-undecanone (SIGMA U1303-5ML) was diluted in ethanol in a series of serial dilutions. Each dilution was plated with a volume of 10 µl onto 7.5 mL of Vogel's media with agar in a 60 mm petri dish. 10 µl of FGSC 4289 conidial suspension of approximately 50 spores/µl was added to each plate. The plates were incubated in 12 hour light and dark conditions at 25°C, and germinated spores were counted. The EC50 growth curves were calculated with AAT Bioquest, <https://www.aatbio.com>.

#Results:

Results: We noticed that when *Serratia marcescens* (strain ADJS-2C_Red, Smar) streaked on the plate across from fungi, fungal growth was repressed. The growth area could quantitatively be assessed, based on growth with bacteria streaked on either side compared to growth with no bacteria (Figure 1A). However, this method is not ideal for studying volatiles because it allowed for bacterial metabolites or proteins to diffuse through the media, potentially coming in direct contact with the fungal hyphae. Additionally, the bacteria are mobile and could possibly induce a response by direct contact with the fungal hyphae. The “donut” assay was developed to limit diffusion of bacterial products in the media to fungal hyphae (Figure 1B). This set-up allows for the exchange of gases between the fungi and bacteria, but prevents direct contact and allows for two types of media to be poured (one tailored to the bacteria and the other tailored to the fungi). This is useful for the use of antibiotics in the fungal media or fungi with special growth needs. We tested the ability of various strains of bacteria and the amount of time they needed to be present on the media before the Rstol were introduced (Figure 2). We tested for this inhibition across many fungi from various phylogenetic clades such as *Basidiobolus*, *Mucor*, *Rhizopus*, *Alternaria*, and *Neurospora*. For all strains the exchange of gases was enough to inhibit fungal growth after two days of bacteria growth. LB media was used to grow bacteria and was left uninoculated in the control experiments. The fungal spores/conidia were introduced onto the center plate after 48 hours of bacteria growth. The plates were measured after 24 and 48 hours, (Figure 3). The results show that Spro followed by Smar then Bsub had the highest inhibition of Rstol growth. Spro inhibition of Ncrassa and *Rhizopus* Smar had an average growth being 1.5 cm at 24 hours and 4 cm at 48 hours. Bsub was a weaker inhibitor of growth with the average growth of the fungi being 2.8 and 5.2 cm at 24 and 48 hours, respectively. Control growth of Rstol with no bacteria inoculated on the LB outer ring was 3.7 cm at 24 hours and had grown to the diameter of the plate (5.7 cm) at 48 hours. After establishing that fungal growth inhibitors reside in the volatiles of bacteria we used Gas Chromatography coupled with Mass Spectrometry to identify the compounds produced by our strains of bacteria. Based on previous literature

we assumed that we would find 2-undecanone and dimethyl disulfide [30]. On average there were about 50 volatiles produced by each strain. Strains with less than 500ng detected were thrown out, Smar_F and Spro_I, (Figure 4). The volatiles identified are listed in supplemental table 1. To analyze the volatiles, we restricted the results to only the volatiles with a confident prediction (Thermo Mass Spectrometry RSI Score) of over 85%, and volatiles found in more than half of a sample's replicates, this left us with 5-15 unique volatiles produced per species with 13 compounds produced by all the bacteria (Figure 5). Using an internal standard amounts of volatiles were predicted, this left us with an approximate abundance of compounds. Smar, Spro, and Bsub strain had different volatiles as well as different amounts produced (Figure 6). The top quartile of production had 13 compounds produced by two out of three of the species, only Anisole and 2-methoxyphenol were produced by Smar solely (Figure 7). Non Dimensional Multivariate Scaling (NMDS) shows low stress and resolves *Bacillus* and *Serratia* cleanly. To further analyze the data we used MetaboAnalyst [74,75], an R package for metabolite profiles. Comparisons were made between all three samples, Bsub, Smar and Spro to see if each species could be differentiated by volatile profiles. Auto-scaling centered around the volatile production average was used to make all metabolites comparable (Figure 9), the samples were averaged together and compared to show which samples were differentially produced by the bacterial strains (Figure 10). Principal Component Axis (PCA) analyzed the variance between each of the replicates (Figure 11). ANOVA analysis with a p-value cut off .05 and Tukey's/HSD analysis reveals eleven significantly produce volatiles compounds (Figure 12, Table, Figure 13). After 5000 trees Spro and Smar were unable to be predicted based on their profiles (Figure 14).

The analysis identified volatiles produced by Smar, Spro, and Bsub. We tested 2-undecanone, Dimethyl Trisulfide, Anisole, Lepidine, 2,5-dimethylpyrazine as they were some of the highest produced volatiles from the bacteria. 2-undecanone was chosen because it is a known antifungal [30], is used in food and fragrance, as well as an effective insect repellent approved for use with humans [68,76]. Dimethyl Trisulfide was tested based on the abundance in the Spro profile and sulfur content. Anisole was solely produced by Smar in high amounts. To test the volatiles we loaded the compounds onto a piece of filter paper on a glass microscope slide on the side of a petri dish with media, spores/conidia were inoculated approximately 2 cm away on the media. The effective volatiles for preventing growth were 2-undecanone, Anisole, and Dimethyl Trisulfide, figure (Figure 15). Microscopy phenotype with volatiles reflect the growth inhibition results. Vacuolization was found in 2-undecanone and DMTS samples (Figure 16, Microscope Images).

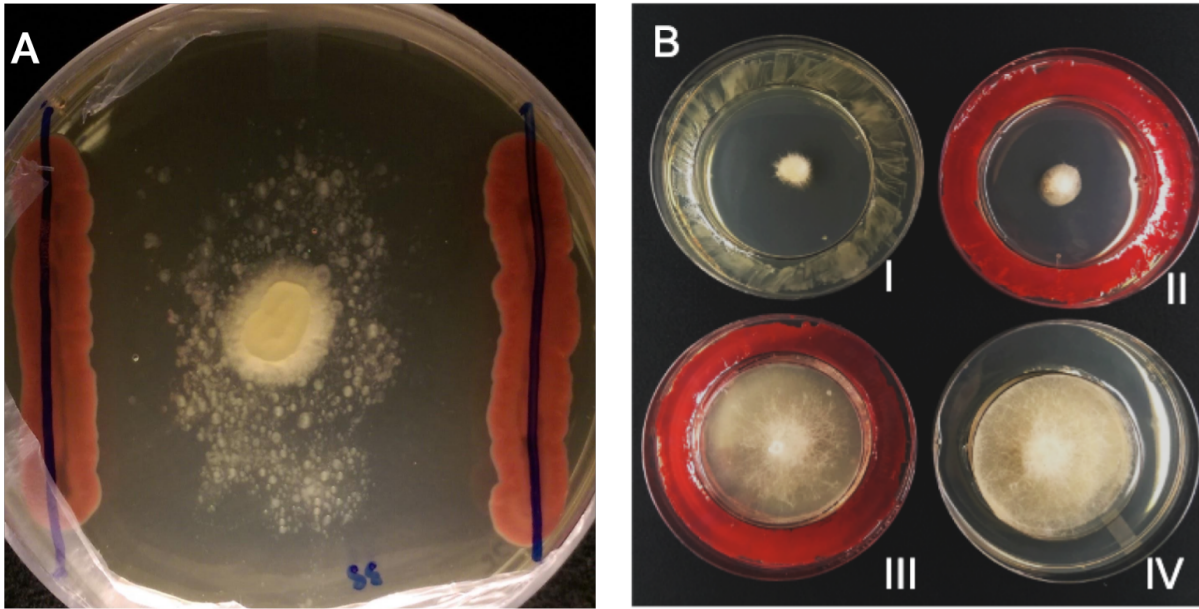


Figure 1: A) Streaks of *Serratia* on either side of fungi. B) Donut plate assays of *Rstol* and other fungi showed the functionality of volatiles inhibiting fungal growth in the presence of bacteria. The bacteria in the LB agar ring on the outside of the plate were not able to come into physical contact with the fungi. Interactions were solely restricted to the volatiles released into the shared headspace of the petri dish. Donut plates both show fungal inhibition of growth with at 48 hours (I *E. coli*, II *Smar*, III *Smar* fungi covered, IV No bacteria).

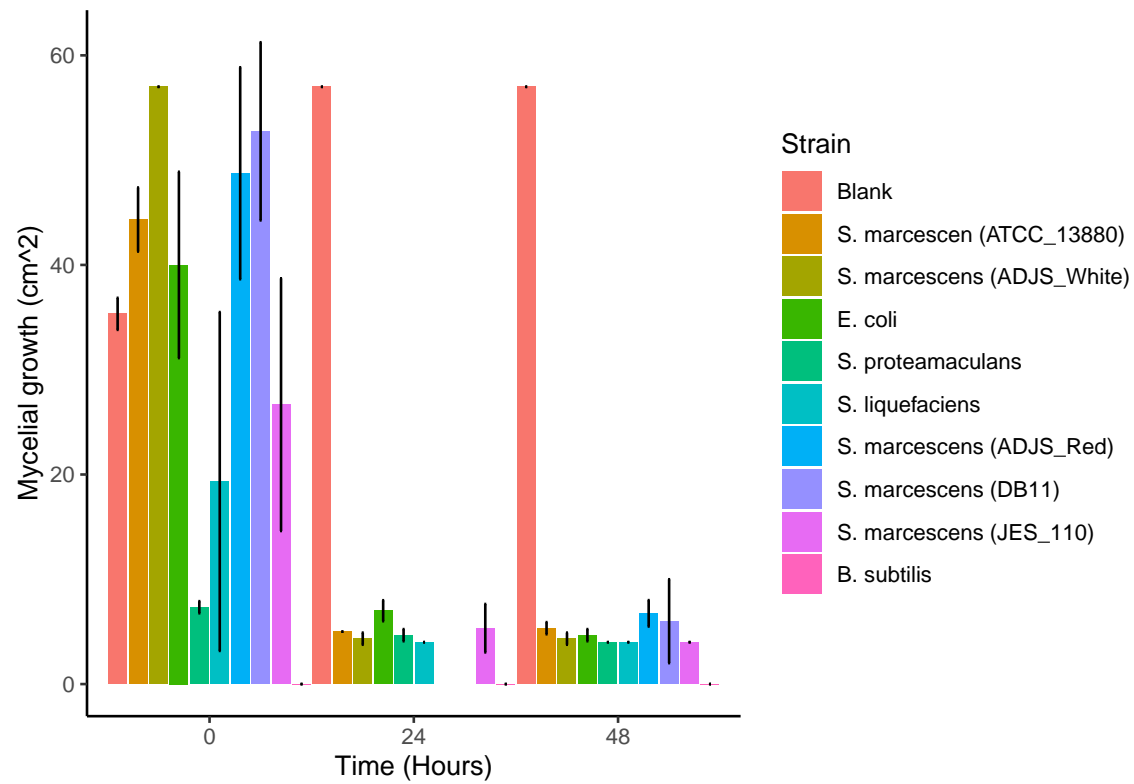


Figure 2: Figure 1: We tested the ability of various strains of bacteria to inhibit fungal growth at 0, 24, and 48 hours

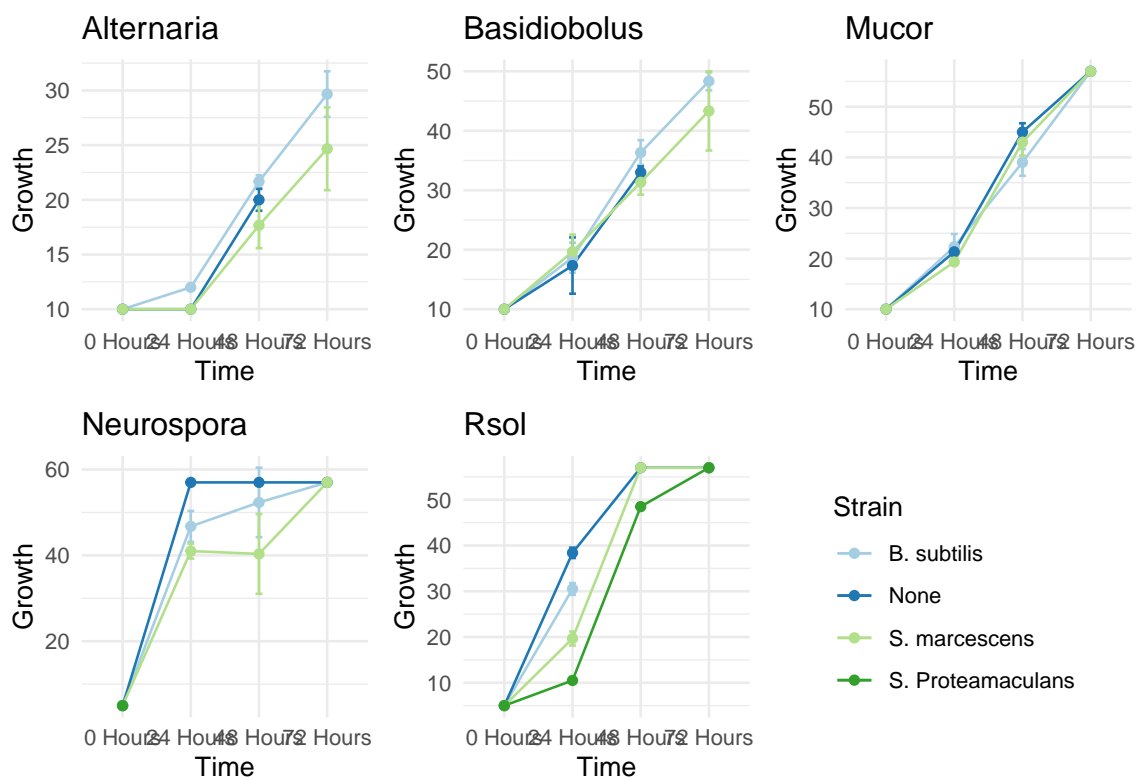


Figure 3: Pathogenic fungi and their growth inhibition with bacterial volatiles

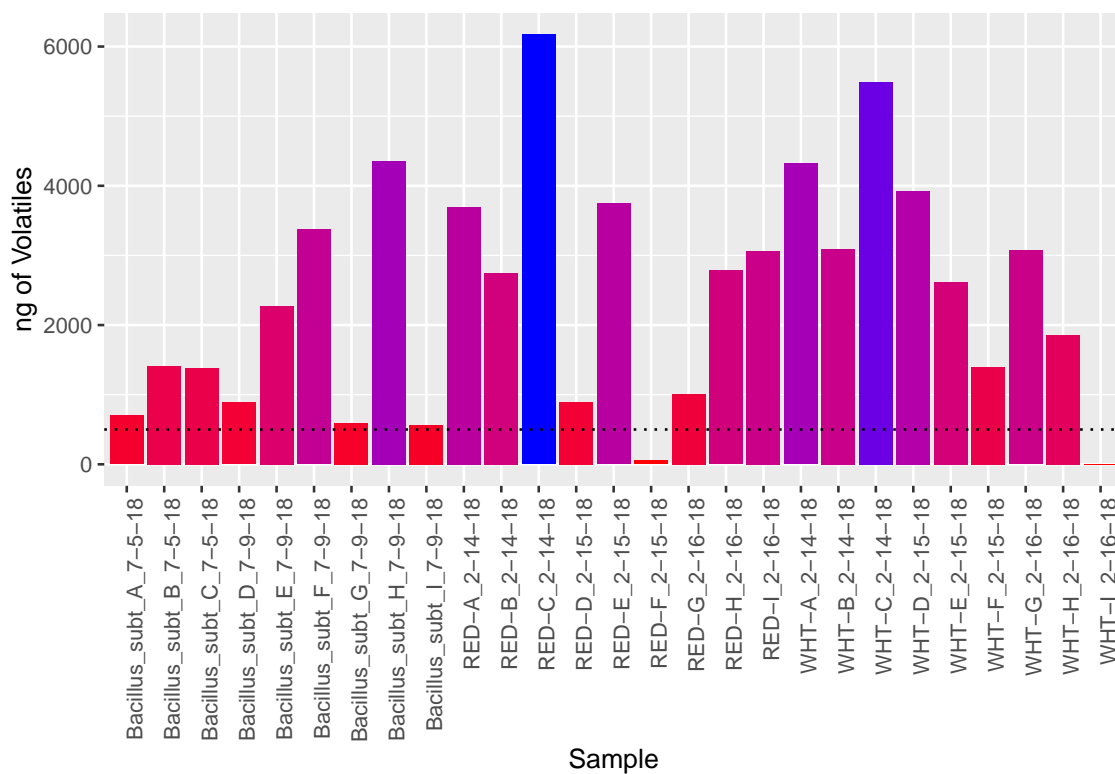


Figure 4: Effect of the most abundance VOCs produced by bacteria on *Rhizopus* growth after 24 hours

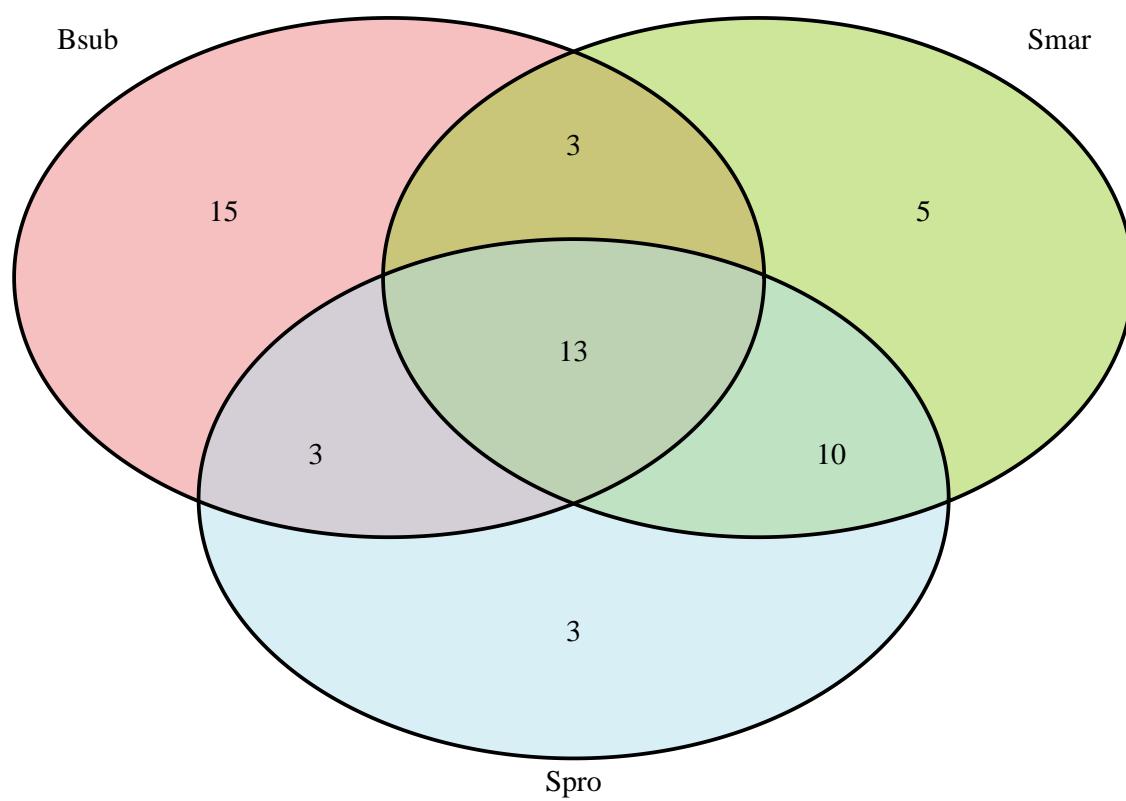


Figure 5: Over lap of related volatiles produced by three species of bacteria

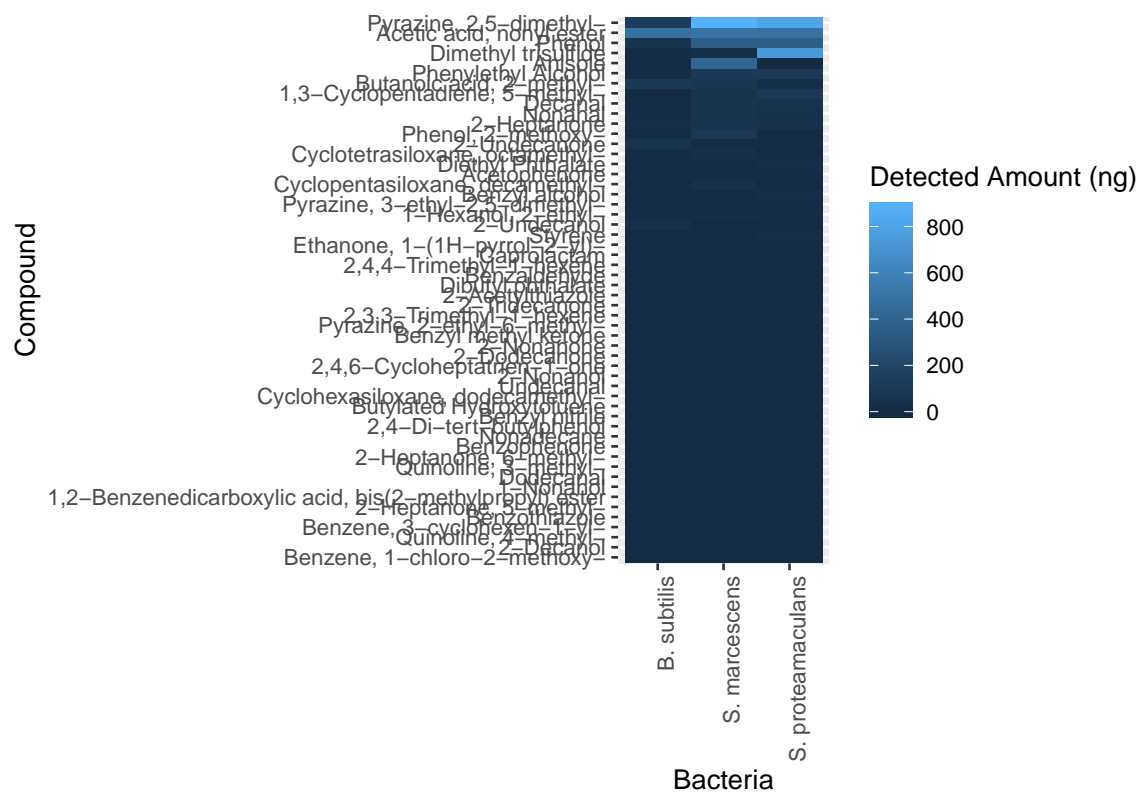


Figure 6: Average volatile production across all samples

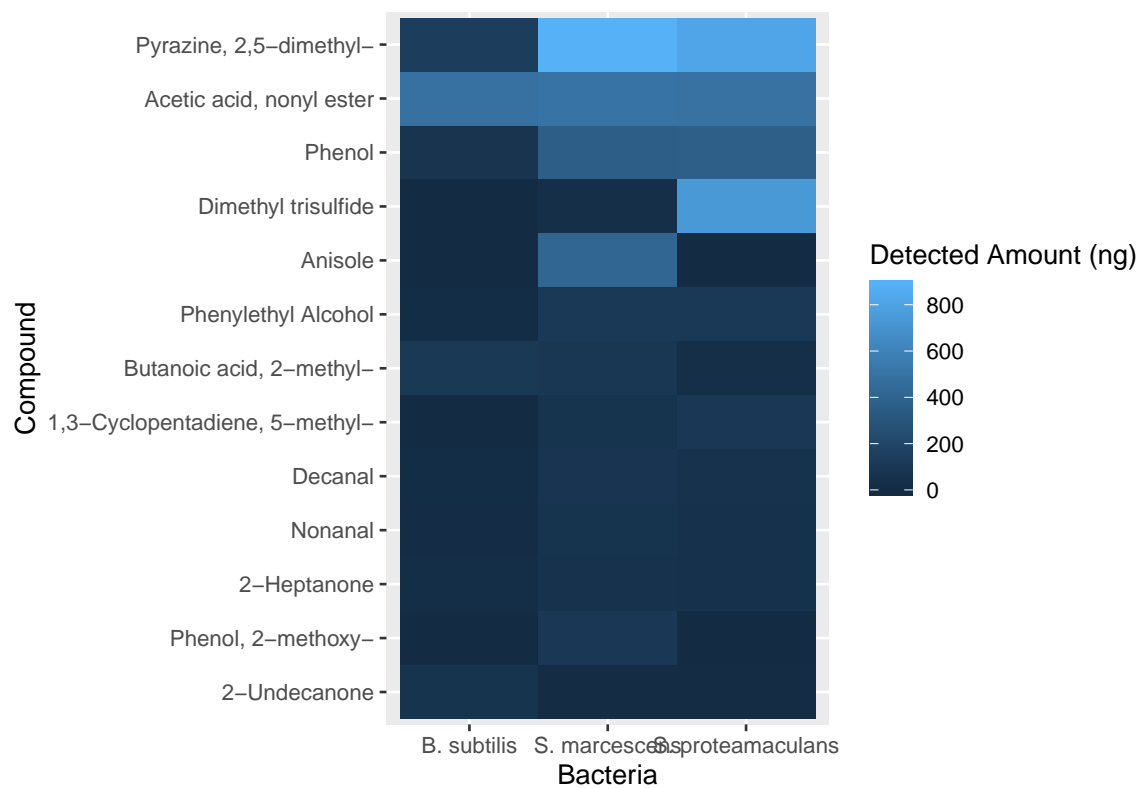


Figure 7: Average volatile production across all samples with

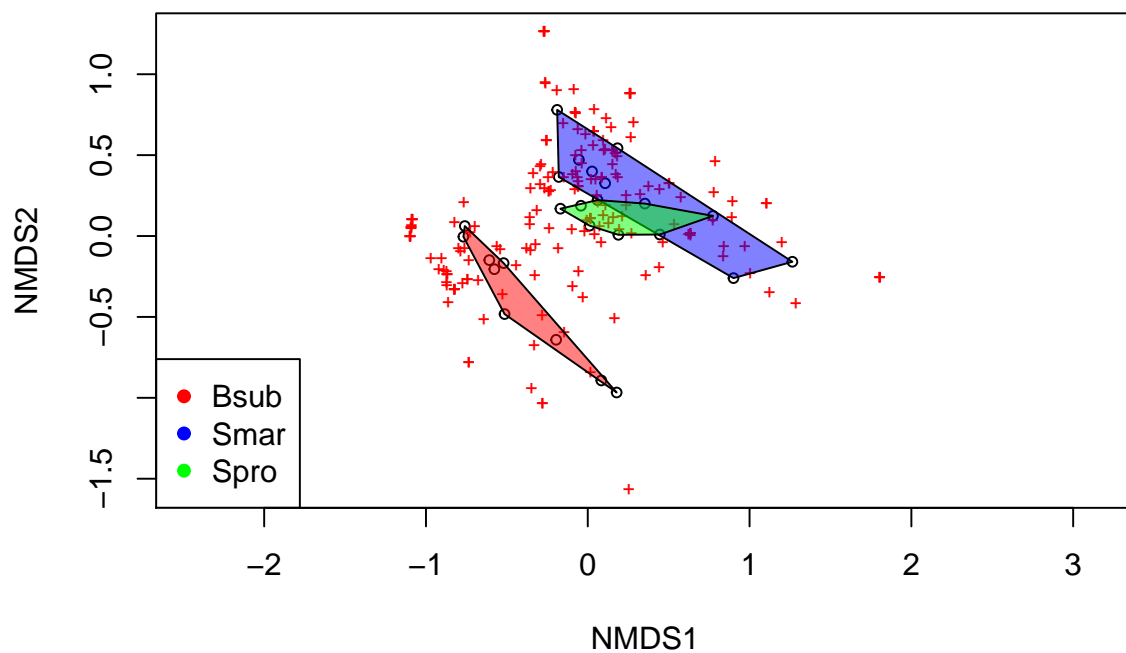


Figure 8: NMDS showing differences between Bacillus and Serratia

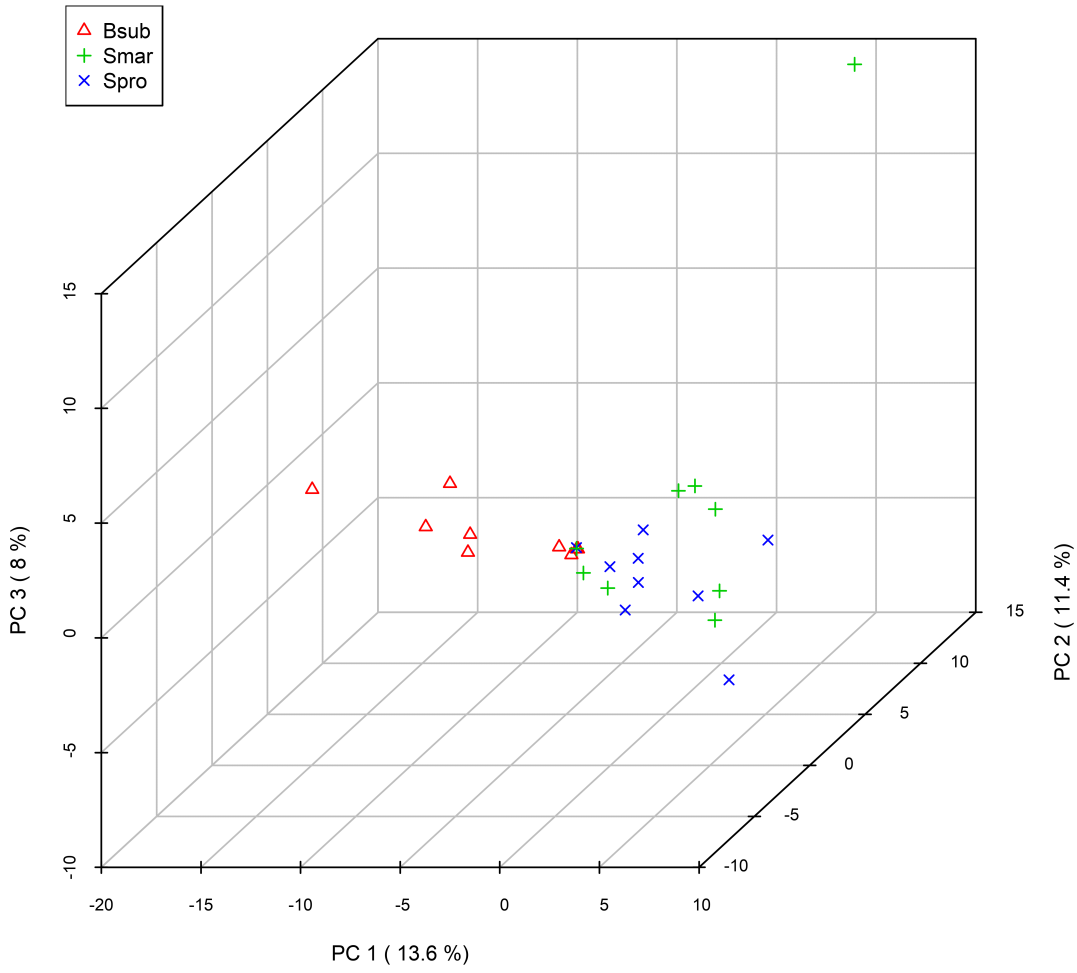


Figure 9: PCA plot showing variation amount samples of Bsub, Smar, and Spro

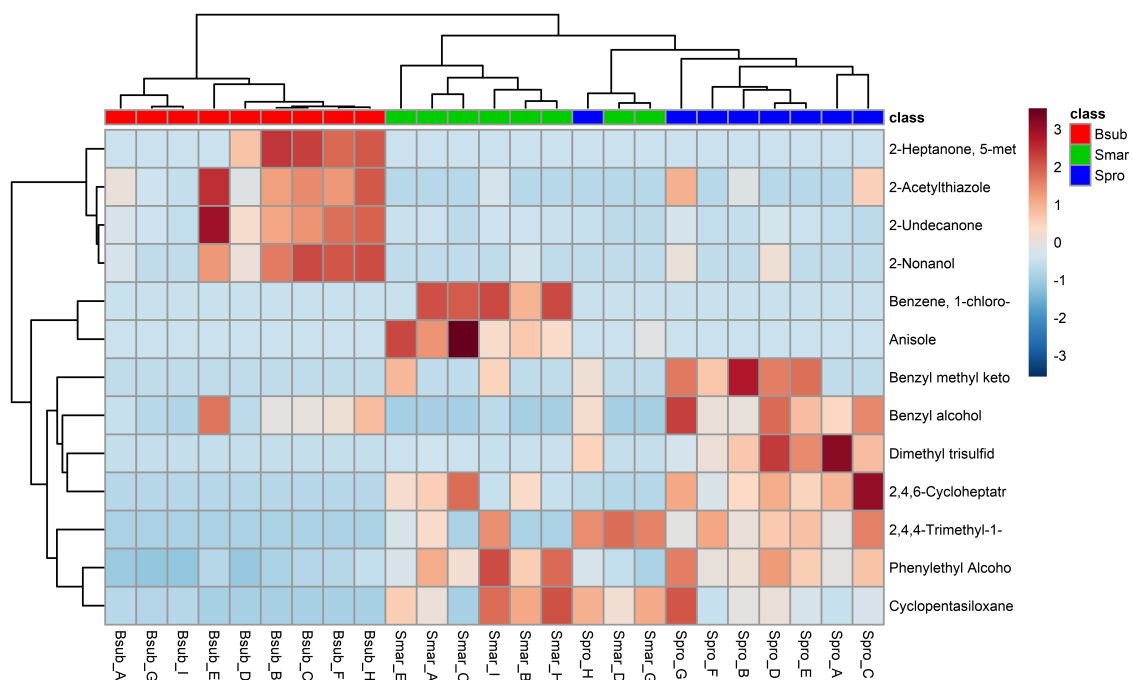


Figure 10: Shows autoscaled volatiles and the production relative to each sample

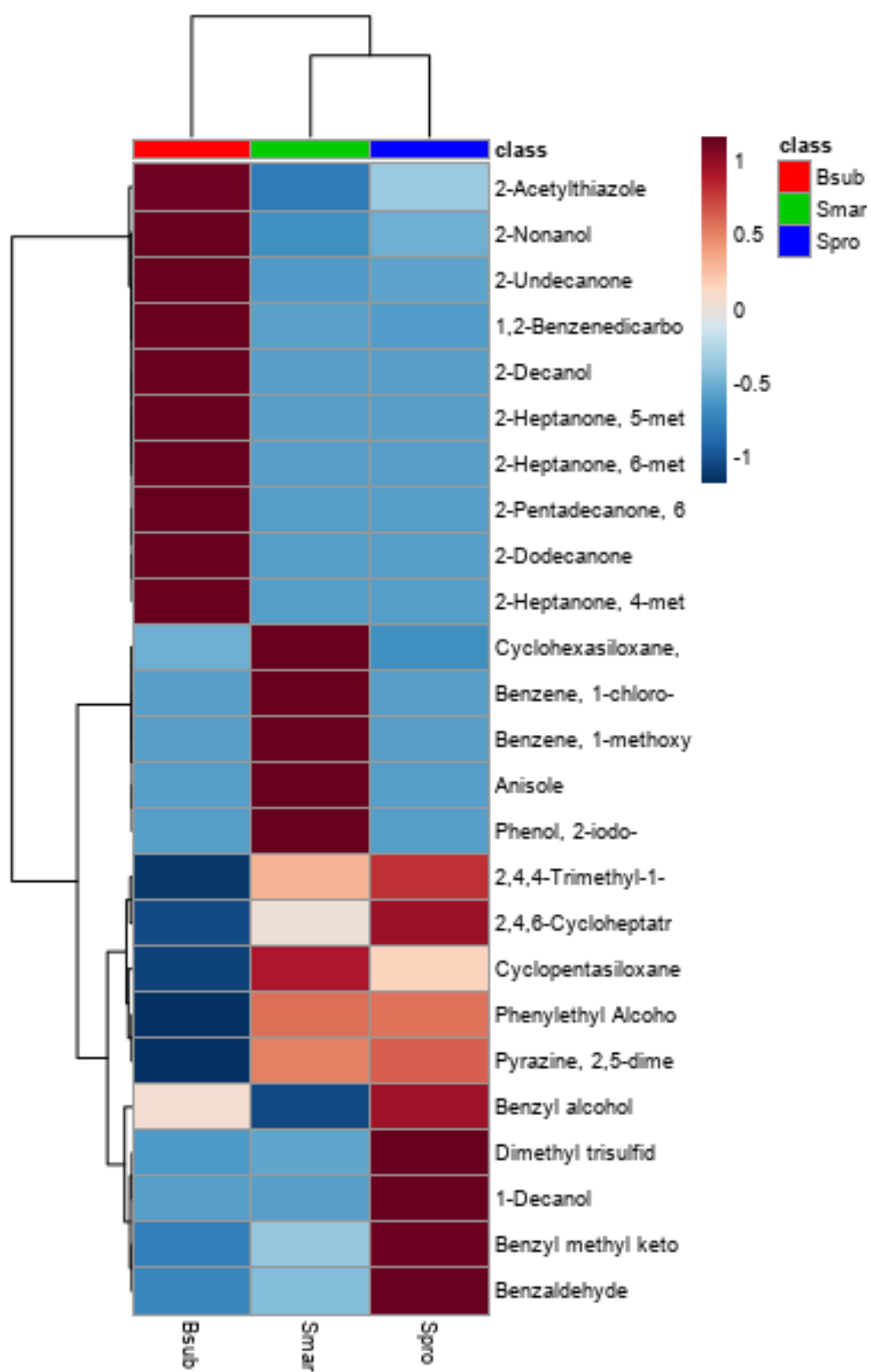
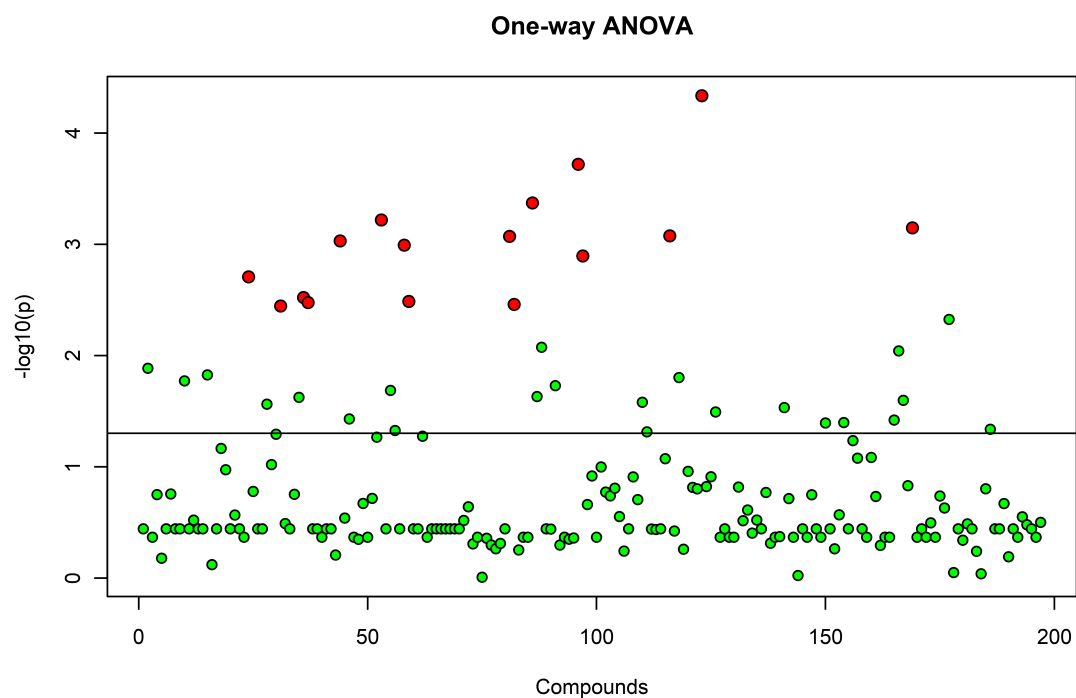


Figure 11: Average of volatiles produced by each species of bacteria



\begin{figure}[H]
\caption{ANOVA results showing 95% confidence or higher 11 compounds differentially produced by Bac-
teria} \end{figure}

| Compound | f.value | p.-value | FDR | Fisher's LSD |
|--------------------------------|---------|-----------|----------|--------------------------|
| Dimethyl trisulfide | 12.3500 | 0.0002052 | 0.021673 | spro - Bsub; spro - smar |
| 2-Undecanone | 12.2390 | 0.0002167 | 0.021673 | Bsub - smar; Bsub - spro |
| 2-Nonanol | 11.2110 | 0.0003647 | 0.024312 | Bsub - smar; Bsub - spro |
| Benzyl alcohol | 9.9184 | 0.0007252 | 0.032698 | Bsub - smar; spro - smar |
| 2-Acetylthiazole | 9.7008 | 0.0008174 | 0.032698 | Bsub - smar; Bsub - spro |
| Benzene,1-chloro-2-methoxy- | 9.1822 | 0.0010928 | 0.036425 | smar - Bsub; smar - spro |
| 2-Heptanone,5-methyl- | 8.6883 | 0.0014503 | 0.037500 | Bsub - smar; Bsub - spro |
| 2-Heptanone,6-methyl- | 8.4896 | 0.0016284 | 0.037500 | Bsub - smar; Bsub - spro |
| 2-Dodecanone | 8.3534 | 0.0017642 | 0.037500 | Bsub - smar; Bsub - spro |
| Anisole | 8.2186 | 0.0019106 | 0.037500 | smar - Bsub; smar - spro |
| Cyclopentasiloxane,decamethyl- | 8.0901 | 0.0020625 | 0.037500 | smar - Bsub; spro - Bsub |

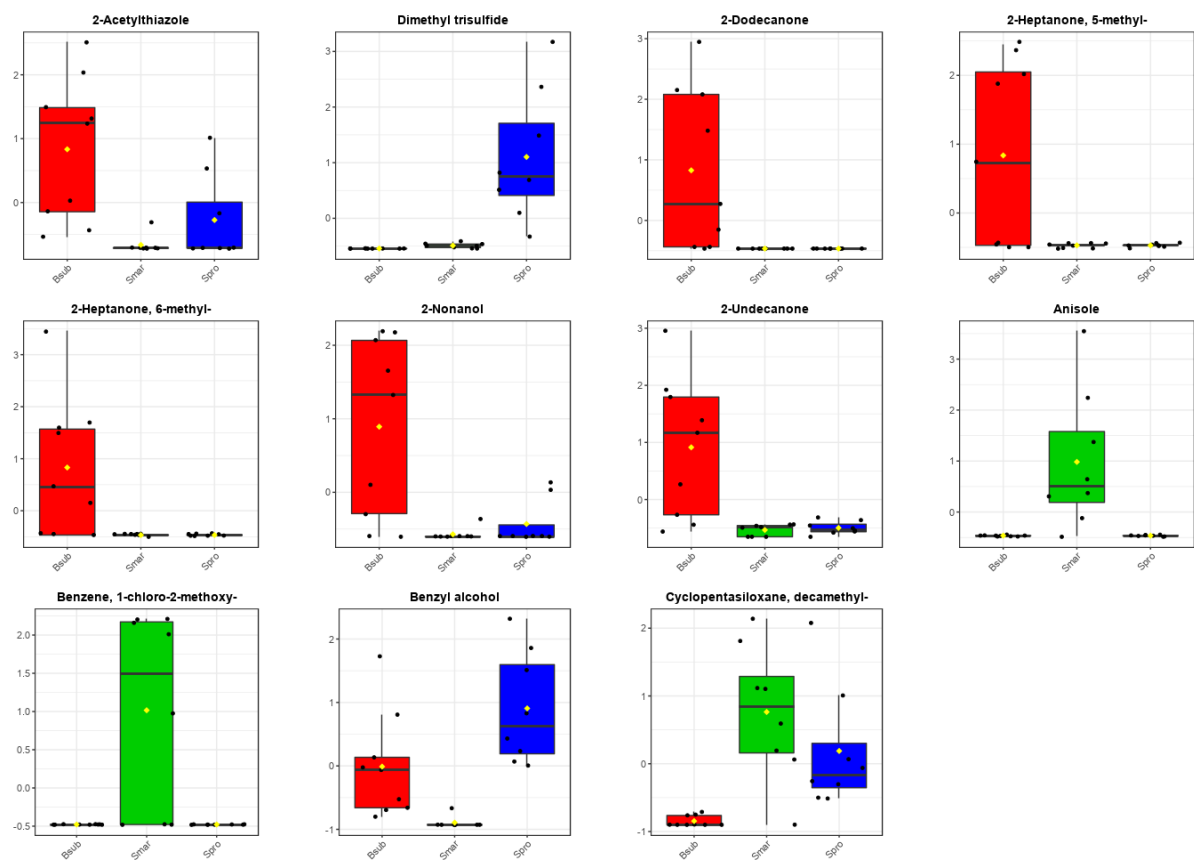


Figure 12: Relationship of differentially produced volatiles between each bacteria species

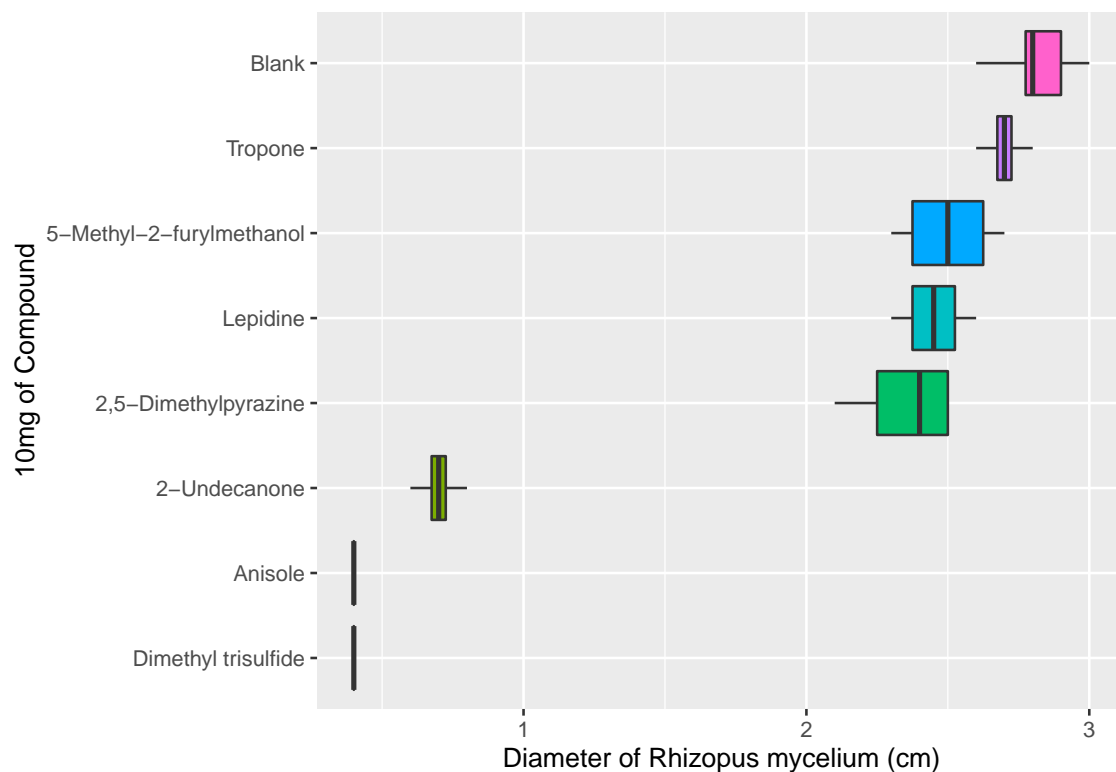


Figure 13: Growth measurements after 24 hours of exposure to 10mg of each compound

Discussion:

The biggest impact on the field. A reexamination of volatiles produced by *Serratia marcescens* reveals several antifungal compounds. An issue with 2-undecanone is that it has a very short halflife of 1.2 days [76]. The context of impact within the field - Many studies are beginning to incorporate microbial communities into their work, the volatile profiles of these communities are at times neglected but can clearly play a major role. Support from other work includes microbial community assembly work is being done, this adds to the body of work showing the importance of fungal bacteria interplay [1]. Limitations of the work are that we are limited to the bacterial profile being created while bacteria are on LB media, hardly a realistic environmental conditions. It would be interesting to see the volatiles produced in various soil types and with various plant partners to see if there is a core volatome.

Conclusion:

It appears the key to resisting the bacteria compounds is to metabolize or neutralize the volatiles. More work needs to be done in other systems, especially outside of a petri dish. Spores/Conidia can sense the presence of volatiles and wait until favorable conditions, this would be an interesting follow-up experiment to learn about environmental cues that stop fungi from germinating from conidia. Looking into additional compounds to find more fungal responses or if there is a conserved fungal response. Anisole would be an interesting compound to follow up on, only found in *Serratia marcescens* (ADJS-Red). Not in *Bacillus*. Searching for new antifungals is important. Looking to the future, volatile profiles with multiple antifungal components may be important for the war against fungi.

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