Secondary metabolites of *Bacillus subtilis* impact soil-derived semi-synthetic bacterial community assembly

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Keywords: *Bacillus subtilis*; non-ribosomal peptides; bacterial community; chemical ecology; *Lysinibacillus fusiformis*; surfactin

Abstract

Secondary metabolites provide *Bacillus subtilis* with an increased competitiveness towards other microorganisms. In particular non-ribosomal peptides (NRPs) have vast antimicrobial potentials by causing cell lysis, perforation of fungal membranes, enzyme inhibition or disruption of bacterial protein synthesis. This knowledge was primary acquired *in vitro* by competing *B. subtilis* with other microbial monocultures. However, our knowledge about the true ecological role of these small molecules is limited.

In this study, we have established soil-derived semi-synthetic mock communities containing 13 main genera and supplemented them with *B. subtilis* P5_B1 WT, its NRPs deficient strain *sfp* or single NRP mutants incapable of producing surfactin, plipastatin or bacillaene. 16S amplicon sequencing revealed that the abundances of two genera, *Lysinibacillus* and *Viridibacillus,* were reduced when *B. subtilis* strains producing NRPs were invading the community. Interestingly, this effect was diminished in communities supplemented with the NRPs deficient strain. Growth profiling of *Lysinibacillus fusiformis* M5 exposed to either spent media of the *B. subtilis* strains or pure surfactin indicates the sensitivity of this strains towards the biosurfactant surfactin.

Our study provides a deeper insight on the influence of *B. subtilis* NRPs on semi-synthetic bacterial communities and helps to understand their ecological role.

Introduction

In nature, bacteria live in complex communities where they interact with various other microorganisms. Microbial communities are among others important for influencing biochemical cycles, agriculture, and plant growth promotion [1–4]. The study of these communities is essential to scrutinize the natural processes taking place in such consortia, to understand their impact on the environment, and to be able to eventually engineer them eventually [5]. To investigate the community members, their functions and interactions such as metabolite cross-feeding interactions, extensive research has been conducted in the last decade [6,7].

One such ecosystem harbouring plenty of microbial communities is the rhizosphere where various interactions between bacteria, fungi and plants take place. The microbial communities are dynamic and dependent on many different factors such as plant species, soil type and season [8–14]. Microbial communities can consist of hundred to thousand different species, which makes investigations very complex and hard to reproduce. One alternative approach is to establish a simpler synthetic community with a defined composition [13,15]. Lebeis *et al.* used a synthetic community of 38 strains to demonstrate that plant phytohormones sculpt the root microbiome, while Niu *et al.* established a seven-species community based on host selection to mimic the principle root microbiome of maize [13,16]. An important mediator of interactions between microorganisms is believed to be secondary metabolites (SMs) [17]. Many of them are well-studied *in vitro*, however the true ecological role of SMs is still subject of investigation. Contradictory opinions exist in literature, some share the view that SMs are mainly microbial weapons, but others instead designate them as signalling molecules [18–21]. Additionally, numerous studies have demonstrated that

microbial communities also impact SM production, since their production is increased in mixed fermentations or otherwise silent gene clusters are only expressed in co- or multi-cultures [22,23].

Bacillus subtilis is a well-studied soil bacterium and is used as a model organism for biofilm formation and sporulation [24]. It has been shown that several members of the *B. subtilis* species complex have exceptional plant-growth-promoting and plant-health-improving properties by suppressing plant pathogenic bacteria and fungi [25]. However, it not completely understood how soil administered *Bacillus* spp. affect the indigenous microbial communities. Gadhave *et al.* have showed that supplementation of different *Bacillus* spp. led to species-dependent changes in the diversity, evenness, and relative abundances of endophytic bacterial communities [26]. Like many other soil bacteria, *B. subtilis* and other *Bacillus* spp. produce various SMs [27,28]. The most prominent and bioactive SMs are non-ribosomal peptides (NRPs), whose isoforms belong to the families of surfactins, fengycins or iturins and are synthesized by large enzyme complexes, non-ribosomal peptide synthetases (NRPS) [29,30]. *B. subtilis* has four *sfp*-dependent gene clusters, three NRPS gene clusters (surfactin, plipastatin and bacillibactin) and one hybrid NRPS-PKS gene cluster (bacillaene). For the synthesis of all these NRPs, the phosphopantetheinyl transferase *Sfp* is needed since it activates the peptidyl carrier protein domains, converting it from the inactive apo-form to the active holo-form [31]. The well-studied biosurfactant surfactin, encoded by the *srfAA-AD* gene cluster, reduces the surface tension needed for swarming and sliding motility [32,33]. Its bioactivity is specifically evoked by the surfactant activity triggering cell lysis due to penetration of bacterial lipid bilayer membranes and formation of ion-conducting channels [29,34–36]. It is presumed that the antifungal plipastatin, expressed from the *ppsA-E* gene

cluster, acts as an inhibitor of phospholipase A2, forming pores in the fungal membrane and causing morphological changes in fungal membrane and cell wall [37,38]. Its antifungal potential primarily against various filamentous fungi was demonstrated [39–43]. The broad-spectrum antibiotic bacillaene, synthetized by the *pksA-S* gene cluster is mainly targeting bacterial protein synthesis but it was also shown that it can protect cells and spores from predation [44,45]. We recently demonstrated that the production of these NRPs varies among co-isolated *B. subtilis* environmental strains due to missing core genes or potentially altered gene regulation highlighting the existing natural diversity of SM production in this species [43].

In this study, we focus on semi-synthetic bacterial mock communities and describe how these are affected by a *B. subtilis* strain that was previously isolated from the same sampling site. We established semi-synthetic mock communities by pre-incubating a soil suspension for 12 h, seeded these cultures with *B. subtilis* strains as co-inoculum, and further incubated them for 48 h before analysis of the bacterial composition. The impact of NRPs on the establishment and composition of the bacterial communities was investigated by NRP mutant-based approaches. We supplemented the pre-incubated soil suspensions with either wild-type *B. subtilis* (isolate P5_B1) [43], its NRPs deficient strain *sfp* or its single NRPs mutants to reveal if the invasion of *B. subtilis* changes the composition of the microbial community and if NRPs are involved. We previously demonstrated that strain P5_B1 produces the NRPs surfactin and plipastatin and has further BGC predictions for the NRPs bacillaene and bacillibactin [43]. 16S rRNA amplicon sequencing revealed that semi-synthetic mock communities could be established containing 13 genera, of which two were negatively affected by the addition of *B. subtilis*. Further growth profiling confirmed the impact of *B.*

subtilis produced surfactin on the growth of a selected *Lysinibacillus* species.

Results

Impact of *B. subtilis* secondary metabolites on taxonomic groups in semi-synthetic mock communities

We established soil-derived semi-synthetic mock communities and supplemented them with *B. subtilis* WT P5_B1, its NRP deficient strain (*sfp*) or its single NRP mutants incapable of producing either surfactin (*srfAC*), plipastatin (Δ*ppsC*) or bacillaene (Δ*pksL*), or kept untreated culture as control (Figure 1). To investigate the impact of *B. subtilis* NRPs on the bacterial community composition, DNA was extracted from the initial soil sample, the 12-h-pre-cultivated soil suspensions and the *B*. subtilis-treated or untreated mock communities co-cultivated for 48 h, and analysed using amplicon sequencing of the V3-V4 region of the 16S rRNA gene. The taxonomic summaries give an overview of the relative abundance of the most abundant genera present in each assay and replicate (Figure 2). Unsurprisingly, the two soil samples differ tremendously from all other samples in both abundance and number of present genera. We determined that *Bacillus* was the most abundant genus in the two soil samples with a relative proportion between 19 and 35 %. Other genera with abundances higher than 2 % were *Sporosarcina* (4 – 11 %), *Candidatus* Udaeobacter (7 – 10 %) and *Gaiella* (3 – 4 %). The communities of the 12 h-pre-cultivated soil suspension showed a lower diversity and consisted primarily of the two genera *Bacillus* (56 – 65 %) and *Acinetobacter* (29 – 34 %). Additional genera with an abundance higher than 1 % were *Lysinibacillus* (1.2 – 3.2 %), *Pseudomonas* (1.0 – 2.2 %) and *Viridibacillus* (0.6 – 1.5 %). The genus richness of the four pre-cultured soil suspensions were between 12 and 18 of in total 21 unique genera.

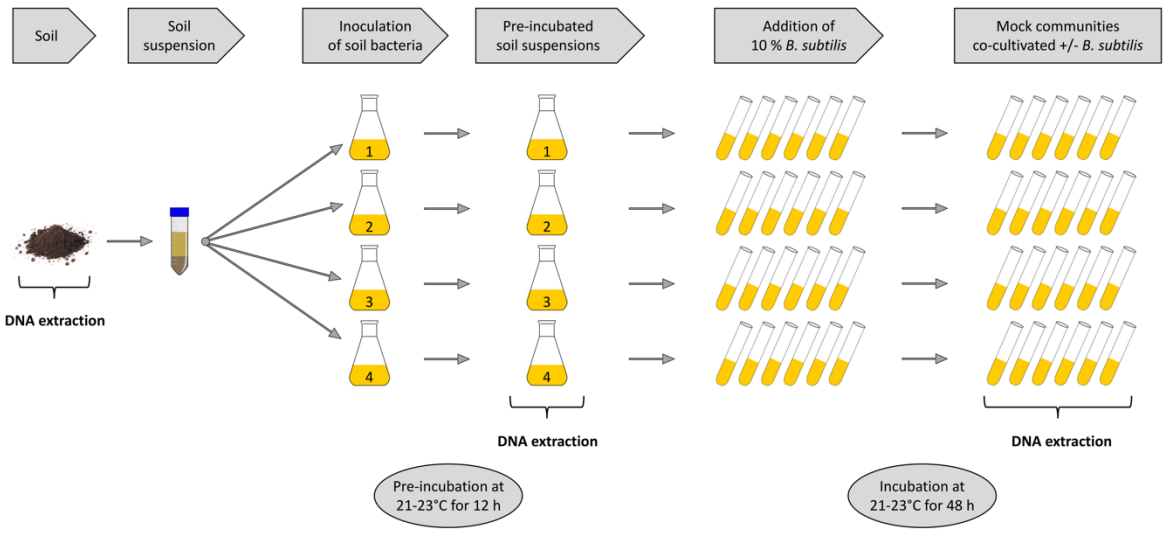

Figure 1. Overview of experimental setup. A soil suspension, obtained from a soil sample was used as inoculum for four independent replicates and pre-incubated for 12 h. Enriched pre-cultures were aliquoted and supplemented with 10 % *B. subtilis* strains or left untreated and incubated for 48 h. DNA was extracted from the soil sample, pre-incubated soil suspensions and mock communities.

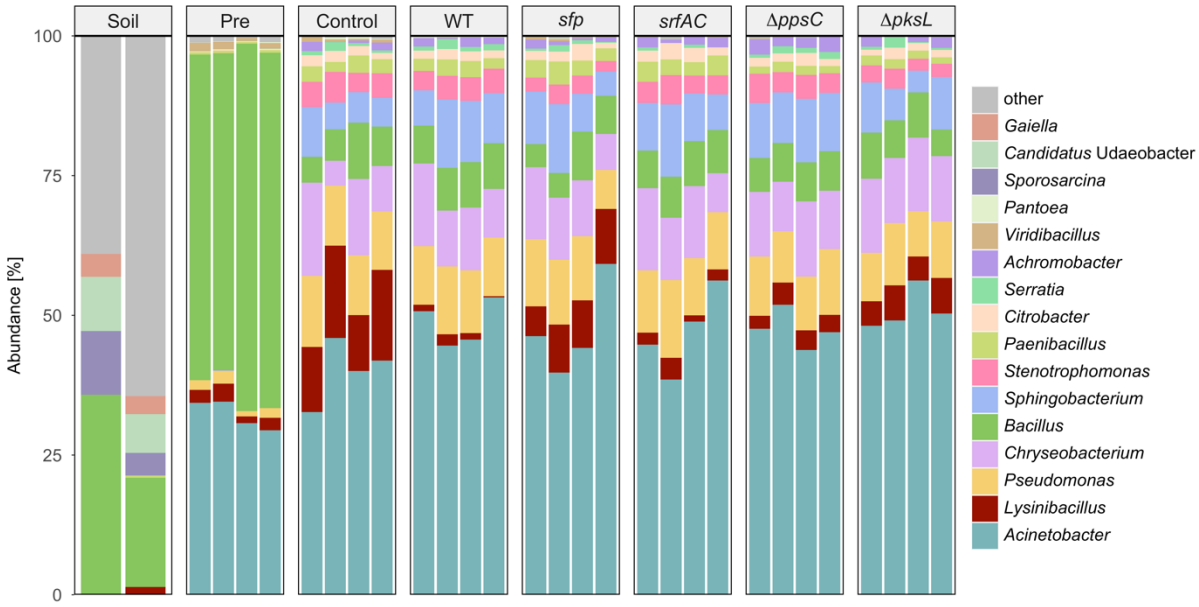

Figure 2. Taxonomic summaries showing the relative abundance of the most abundant genera for each replicate of the soil sample, pre-incubated soil suspensions and different treated mock communities. Genera not present in the majority of the communities or having an abundance < 0.5 % are classified as "other".

To gain a general insight on the impact of NRPs on the diversity of a mock community,

diversity analyses were performed. We excluded the sample Soil 1 from the analysis since it had the lowest read number and its rarefaction curve was not reaching a clear asymptote. The alpha diversity given by the Shannon index revealed a high similarity of the mock communities cultivated for 48 h (Figure 3A). However, it also highlighted that the pre-cultivated communities had the lowest Shannon index, consequently having a lower species evenness and are dominated by fewer species. The soil sample had the highest Shannon index and highlights that richness and evenness is expectedly higher than in the *in vitro* communities. Due to the high differences in alpha diversity of the soil and preincubated samples, we decided to determine the beta diversity based on the Bray-Curtis dissimilarity only for the mock communities cultivated for 48 h to get a more detailed insight on the impact of NRPs. The analysis underlined a high similarity of the different treated mock communities, but the control mock communities were separated from the majority of the other communities along the MDS1 axis (Figure 3B). Furthermore, two replicates of the *sfp*-treated communities showed a high similarity to the replicates of the control communities. In contrast, the other communities supplemented with *B. subtilis* strains exhibited higher similarities to each other. Notably, the communities treated with the *srfAC*-mutant had a higher dispersion, likely owing to a low number of reads in two of the replicates. The alpha diversity analyses revealed that species richness and evenness was reduced in the *in vitro* communities compared to the soil community. Furthermore, communities cultivated for only 12 h showed reduced diversity compared to the mock communities incubated for 24 hours. Nevertheless, we could not detect an effect of the supplemented *B. subtilis* strains on diversity. However, the beta diversity results suggested that the addition of NRPs-producing *B. subtilis* strains influenced the composition of the mock communities.

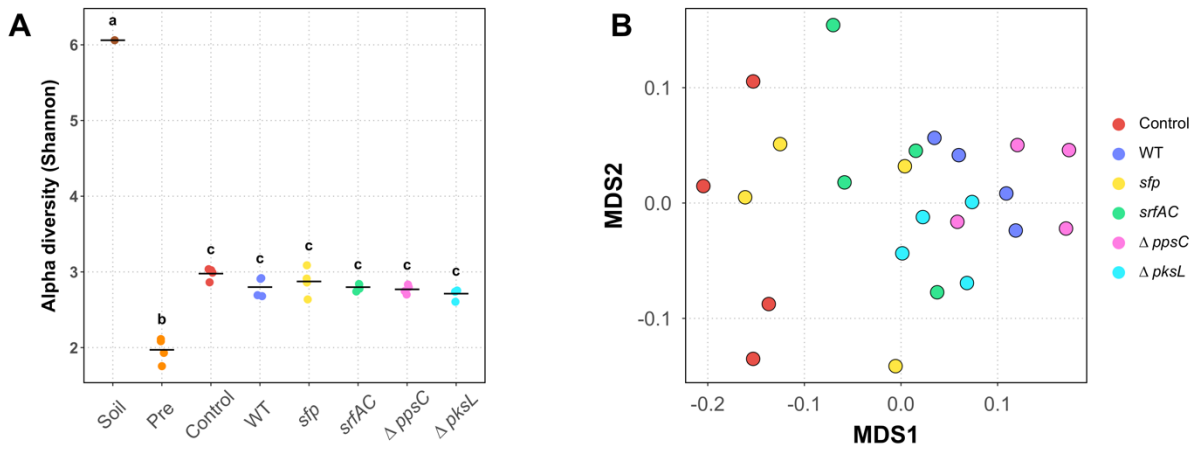

Figure 3. (A) Alpha diversity (Shannon) of the different samples, each point represents a replicate while the line indicates the mean of the Shannon diversity indexes. (B) Beta diversity of the mock communities calculated with the Bray–Curtis dissimilarity and visualized with nonmetric multidimensional scaling (NMDS).

The diversity, in particular the species evenness, increased in all established mock communities independent of the treatment compared to the pre-cultivated soil suspensions and contained 11 - 18 different genera (Figure 2). The most abundant genera having a proportion greater than 1 % in the *B. subtilis*-treated or untreated mock communities were *Acinetobacter*, *Lysinibacillus*, *Pseudomonas*, *Chryseobacterium*, *Bacillus*, *Sphingobacterium*, *Stenotrophomonas*, *Paenibacillus*, *Citrobacter*, *Serratia*, *Achromobacter*, *Viridibacillus* and *Pantoea*. Noteworthily, the prevalence of the *Bacillus* genus was comparable in the *B. subtilis*-treated communities (4 – 9 %) and the control (5 – 10 %), in which *Bacillus* could only originate from the soil suspension. Comparisons of abundance ratios between the control communities and *B. subtilis* WT-treated communities revealed that *Lysinibacillus* and *Viridibacillus* were significantly decreased 10 and 8.3-fold, respectively, in the communities supplemented with *B. subtilis* WT (Figure 4A). None of the other genera were negatively affected by the addition of this strain. In comparison, we could only detect a 1.8-fold reduction of *Lysinibacillus* in the *sfp*-treated communities compared to the untreated communities, thus greatly diminished

effect compared to the WT treated samples (Figure 4B). In addition, we could not observe significant reduction of *Viridibacillus*, but *Stenotrophomonas* was likewise 1.7-fold reduced besides *Lysinibacillus* in these communities. The direct comparison of WT- and *sfp*-treated communities confirmed the NRPs-dependent suppression of both *Lysinibacillus* and *Viridibacillus* in the WT-treated communities and the suppression of *Stenotrophomonas* in the *sfp*-treated communities (Supporting Information File 2, Figure S1).

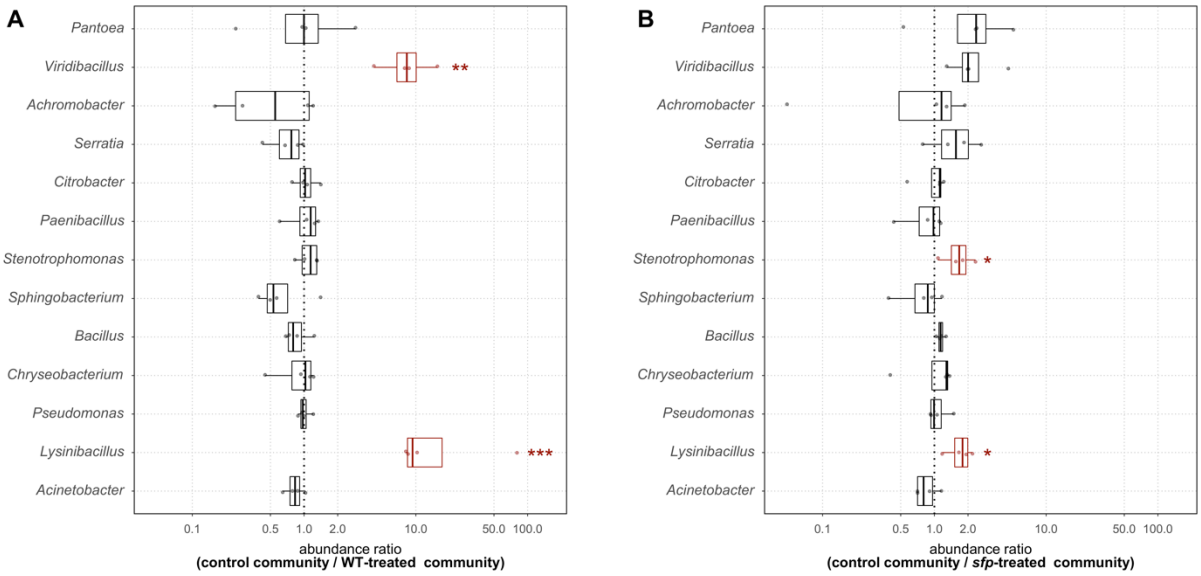

Figure 4. Abundance ratios for each genus and replicate (points) in the (A) control community compared to the WT-treated community and (B) control community compared to the *sfp*-treated community. Statistical significance is defined as P ≤ 0.05 (*), P ≤ 0.01 (**) and P ≤ 0.001 (***).

Concentrating on *Lysinibacillus*, the highest abundance of this genus was discernible in the control assays (13.9 %), which were significantly different compared to all other *B. subtilis*-treated assays (Figure 5). However, when *B. subtilis* WT was added to the mock communities, a significant decrease of *Lysinibacillus* (1.2 %) was discovered. Furthermore, when we added the NRPs deficient strain *sfp* we could notice a significant higher abundance of *Lysinibacillus* (8.6 %) compared to the WT-treated assays, but still a significantly lower prevalence

compared to the control assays. The frequency of *Lysinibacillus* was slightly but not significantly higher in the communities treated with the single NRP mutants *srfAC* (2.1 %) and Δ*ppsC* (3.3 %) compared to the WT-treated communities. *Lysinibacillus*' abundance in the assays containing the Δ*pksL* strain (5.3 %) was significantly higher than in the WT-treated assays but had no significant differences as compare with the Δ*ppsC* or *sfp*-treated communities. In summary, *Lysinibacillus* was affected by the addition of *B. subtilis* independent of the produced NRPs, but if *B. subtilis* strains capable of producing them were present, the impact on *Lysinibacillus* was enhanced. Furthermore, it seems that bacillaene had the strongest and surfactin the weakest effect on *Lysinibacillus* in the mock communities. The second genus affected by the addition of *B. subtilis* was *Viridibacillus*, which had compared to *Lysinibacillus* a very low abundance in the control mock communities (0.5 %) (Supporting Information File 3, Figure S2). However, when *B. subtilis* WT was added this genus' abundance was significantly reduced (0.03 %), notably, it was below the detection level in two of the WT-treated community replicates. Nevertheless, the abundance of *Lysinibacillus* genus in the *sfp*-treated communities was statistically not significant in comparison to the WT and the control communities. Furthermore, the addition of the single NRP mutants *srfAC*, Δ*ppsC* and Δ*pksL* resulted in communities with *Viridibacillus* frequencies similar to the WT-treated communities (0.08%, 0.05% and 0%, respectively). *Viridibacillus* such as *Lysinibacillus* was affected by the addition of *B. subtilis* to the communities. However, no particular NRP could be assigned to the reduced frequency of *Viridibacillus*.

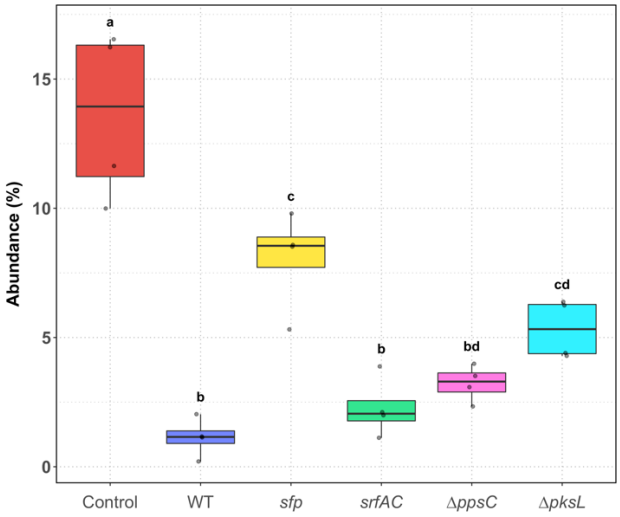

Figure 5. Abundance of *Lysinibacillus* in each mock community. Points represent the abundance in each replicate. Treatments with the same letter are not significantly different.

Growth properties of *L. fusiformis* M5 supplemented with *B. subtilis* spent media

The main finding from the semi-synthetic mock community experiment indicated that the genus *Lysinibacillus* was negatively affected by addition of *B. subtilis* P5_B1 WT and that NRPs enhance the suppression. To dissect the direct impact of particular NRP in this inhibition, we monitored the growth of *L. fusiformis* M5, a previously isolated *Lysinibacillus* species [46], over a time period of 24 h treated with different proportions of spent media from *B. subtilis* WT and its NRPs mutants (Figure 6). When we added about 50 % spent medium to *L. fusiformis,* we observed the best growth in the untreated assay. Interestingly, the addition of spent medium of either WT, Δ*ppsC* or Δ*pksL* caused a growth delay of *L. fusiformis* for the first 13-14 h. Such a strong effect was not observed when the spent medium of the *sfp* or *srfAC* mutant was added. The growth of *L. fusiformis* was only slightly inhibited by these two spent media, although *sfp* spent medium had a lower effect on *L. fusiformis* compared to *srfAC* spent medium. When 23.00 % spent medium was added, no growth differences could be detected anymore between the control and *sfp*-treated assays in the exponential growth phase. Furthermore, the effect of WT spent medium seems to be reduced at this

concentration, but the spent media of Δ*ppsC* and Δ*pksL* maintained their growth inhibition potential. The lowest concentration of spent media having an inhibitory effect was 10.02 %. At this concentration only the spent media of Δ*ppsC* and Δ*pksL* affected the growth of *L. fusiformis* even though it was weakened compared to higher concentrations. Intriguingly, a higher level of aggregation was observed in the *L. fusiformis* assays supplemented with spent medium of *sfp* compared to the other assays, which caused higher and variable OD measurements in the stationary phase of the growth curves (Supporting Information File 4, Figure S3). Finally, we noticed that the final cell densities were slightly higher in the assays supplemented with spent medium than in the control assays.

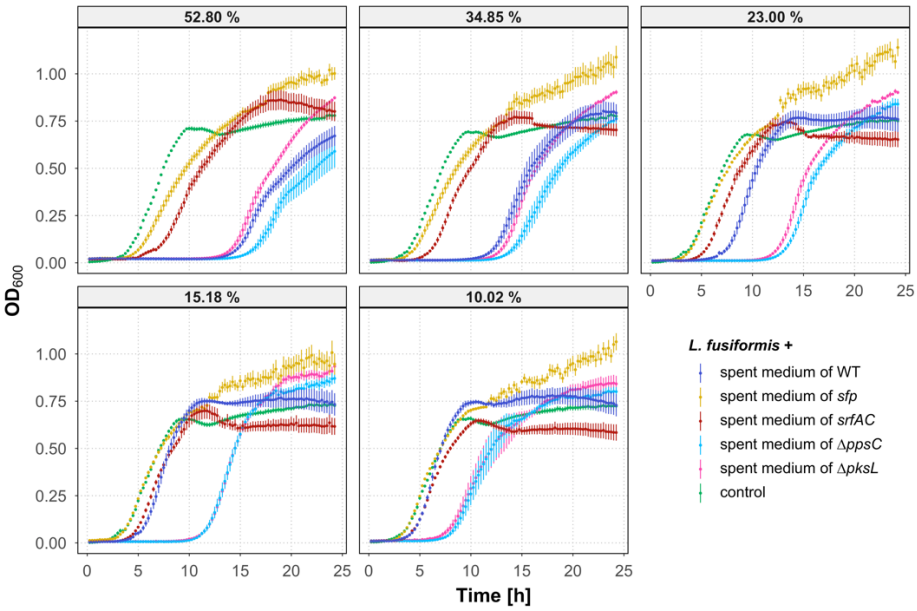

Figure 6. Growth curves of *L. fusiformis* M5 exposed to different concentrations of spent media from the *B. subtilis* strains and without treatment (control). Error bars represent the standard error. N ≥ 6.

These results revealed that *B. subtilis*-mediated inhibition of *L. fusiformis* is NRPs dependent, since the spent medium of the NRPs deficient strain *sfp* had only minor impact. Moreover, we hypothesize that surfactin is responsible for the direct inhibitory effect on *L. fusiformis*, as this

was the only spent medium of a NRP mutant strain with lowered inhibition compared to other spent media of single NRP mutants.

Impact of surfactin on the growth of *L. fusiformis*

To confirm the inhibitory effect of surfactin on *L. fusiformis*, different concentrations of pure surfactin dissolved in methanol were added to the strain and its growth was monitored over a time period of 24 h. Growth of *L. fusiformis* was delayed in the exponential growth phase when surfactin was supplemented in concentrations between 31.25 and 500 µg/ml (Figure 7). At a concentration of 500 µg/ml surfactin, the cell density in the stationary phase was lower than the control. At a concentration of 250 µg/ml the cell density reached a level similar to the untreated control. However, when surfactin in concentrations between 125 and 31.25 µl/ml were added, after an initial growth delay in the exponential phase, the cell densities in all treatments exceeded the ones of the control. The highest concentration of the solvent methanol (5 %) had only a minor inhibiting effect on *L. fusiformis* whereas lower concentrations of methanol showed no inhibition (Supporting Information File 5, Figure S4). These results suggest that surfactin has growth inhibitory effects on *L. fusiformis* and we hypothesize that it might act as the key inhibitory *B. subtilis* NRP under the tested conditions.

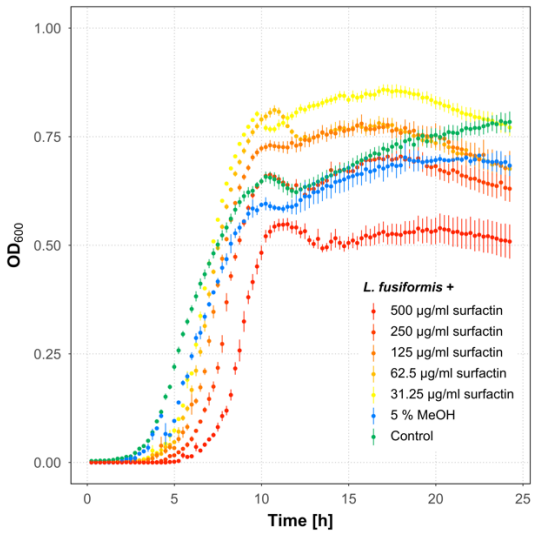

Figure 7. Growth curves of *L. fusiformis* M5 exposed to different concentrations of surfactin, the highest concentration of the solvent MeOH and without treatment (control). Error bars represent the standard error. N ≥ 5 (control and surfactin-treated assays), N = 2 (MeOH-treated assays).

Discussion

B. subtilis is known to produce a wide range of different SMs, which target a large number of different micro and macroorganisms [29]. Our study demonstrates that the NRPs produced by the recently isolated environmental strain of *B. subtilis* P5_B1 influences the composition of a soil-derived semi-synthetic mock community and reveals that *L. fusiformis* M5 is directly affected by the *B. subtilis* lipopeptide surfactin in a monitored growth experiment.

Alpha diversity analyses based on the Shannon estimation revealed that diversity was strongly reduced in *in vitro* cultivations. Furthermore, it disclosed that the pre-cultured soil suspension had the lowest diversity index, due to the fact that mainly the genera *Bacillus* and *Acinetobacter* were enriched which can be probably traced back to different growth rates among the present species. The Shannon index showed no differences among the established and differently treated mock communities, which primary consisted of 13 genera. Even though *Bacillus* was the most abundant genus in the pre-cultures, further incubation for 48 h resulted in decreased relative abundance independently if the respective *B. subtilis* strains were seeded or the pre-cultures were untreated. It shows that the initial dominance of *Bacillus* could not be maintained at prolonged incubation. The *B. subtilis* strains were added at a community assembly phase when *Bacillus* was the dominating genus, so that the general genera distribution was not expected to be influenced extensively. Nevertheless, the final relative abundance of the *Bacillus* genus was not increased after 48 h co-cultivation independent of the treatment, therefore, the presence or absence of NRPs had no impact on its competitive obstruction. However, interactions and competitions within the *Bacillus* genus might still exist and the precise composition within the genus might be distinct among the treatments. Nonetheless, the alpha diversity analysis indicated a higher dissimilarity between the untreated and treated mock communities. In addition, two of the *sfp*-treated

communities showed the highest similarity to these untreated communities suggesting that the supplementation of wild-type *B. subtilis* affected the communities while the addition of the NRPs deficient strain *sfp* was most similar to untreated communities.

Even though microbial interactions and relations will increase with the number of different community members and becoming increasingly complex, we could still observe statistically significant reductions of the two genera, *Lysinibacillus* and *Viridibacillus*, in communities supplemented with the *B subtilis* wild type strain. When supplemented with the *sfp* mutant *Lysinibacillus* was more frequent relative to the wildtype, which indicates that, although it might not be the only factor, NRPs have a great impact on suppressing *Lysinibacillus* abundance. Moreover, no particular NRP could be allocated to the inhibition of the *Lysinibacillus* genus in these semi-synthetic communities, but bacillaene displayed probably the biggest impact on the suppression. A suppression of *Viridibacillus* was also observable and affected by NRPs, but in this case bacillaene had the lowest impact. However, these results must be interpreted with caution and need further investigations, since *Viridibacillus* was one of the lowest abundant genera in the mock communities and abundance calculations are sensitive to the depth of sequencing. Besides the suppression of *Lysinibacillus* and *Viridibacillus*, *Stenotrophomonas* was uniquely suppressed only in the communities supplemented with the *sfp* mutant but not when the WT strain was added. This might be evoked by inhibiting other species which, in turn, has lessened inhibition of *Stenotrophomonas*.

To investigate if *Lysinibacillus* is sensitive to any particular NRP of *B. subtilis*, we exposed a *L. fusiformis* isolate to the spent media of the respective *B. subtilis* strains containing the produced NRPs and monitored the growth. *L. fusiformis* M5 has been isolated from soil and demonstrated to impact biofilm colony development of *B. subtilis* [46]. Interestingly, the

modulation of biofilm development was mediated by the primary metabolite hypoxanthine secreted by *L. fusiformis*. Of note, the impact of *B. subtilis* was not noticed on *L. fusiformis* in the mixed colony biofilm communities, possibly due to the use of a NRPs negative *B. subtilis* strain, 168 that harbours a spontaneous frame shift mutation in *sfp* gene [46]. Testing the impact of the natural isolate *B. subtilis* P5_B1 and its NRP mutant derivatives revealed that the spent media from both the NRPs deficient strain *sfp* and the surfactin deficient strain *srfAC* had the lowest impact on the growth of *L. fusiformis*. In addition, the spent media of Δ*ppsC* and Δ*pksL* maintained their bioactivity at low concentrations, whereas the effect of WT was already strongly reduced at this level of the spent medium. This could be, on the one hand, due to higher levels of surfactin in the two mutants compared to the WT. On the other hand, the spent medium originated from cultures with an optical density at 600 nm (OD_{600}) of 3.0. Cultures with higher ODs were diluted before the harvesting and WT cultures exhibited overall the highest ODs among the strains. Since the NRPs concentration is not proportional to the final OD due to e.g. occurring of cell lysis, the spent media might be slightly differently diluted among the strains. Therefore, minor differences might be observable in the assays supplemented with highly diluted spent media. The observation that *L. fusiformis* displays slightly higher cell density when bacterial spent medium is supplemented might be due to the availability of additional nutrients. Nevertheless, the supernatant and pure compound supplementation demonstrated that surfactin is a direct suppressor of *L. fusiformis*. However, as the spent media of the *sfp* and *srfAC* strains still had growth inhibition effect, it is plausible that next to surfactin further NRPs and even other compounds might provoke slight growth suppression of *Lysinibacillus*.

When *L. fusiformis* was exposed to surfactin concentrations between 125 and 31.25 µl/ml, higher final cell densities were detectable than in the control or in assays treated with higher

levels of surfactin. Interestingly, Arjes *et al.* recently demonstrated that surfactin enhances availability of oxygen for *B. subtilis* by increasing oxygen diffusivity [47], which might also positively affect growth of *L. fusiformis*.

Experiments with differently treated semi-synthetic mock communities have demonstrated that *Lysinibacillus* and *Viridibacillus* were affected by the addition of a NRPs producing *B. subtilis* strain. *Lysinibacillus* was least affected in the mock communities supplemented with the *B. subtilis* Δ*pksL* strain incapable of producing bacillaene, suggesting that bacillaene is the most active compound against this genus. In contrast, the growth curve experiments showed that *L. fusiformis* M5 is most sensitive to surfactin. Importantly, our analysis does not reveal which *Lysinibacillus* species were present in the mock communities, and therefore their sensitivity might be different from the test species used, *L. fusiformis*. Moreover, the spent medium was harvested from pure cultures of *B. subtilis* grown in undiluted complex medium which might change the production of NRPs due to lacking impact of the community members and the level of nutrients. Thus, lower levels of the NRPs in the mock communities might affect *Lysinibacillus* differently compared to the mono-culture growth experiments supplemented with spent media. Finally, *Lysinibacillus* can also be affected indirectly by *B. subtilis* NRPs in the mock communities. Bacillaene is described as an wide-spectrum antibiotic disrupting the protein synthesis in bacteria [28,44]. Even though, the exact mechanisms of it needs to be deciphered, it might have the biggest impact on specific members of the mock community and consequently an indirect effect on *Lysinibacillus*. Interestingly, these two genera, *Lysinibacillus* and *Viridibacillus*, of the mock communities are, besides *Paenibacillus*, the closest relatives of *B. subtilis*. The fact that suppression effects are only observable for these genera, could presumably be caused by the higher overlap in the ecological niches, triggering competition for the same nutrients. Indeed, a higher

phylogenetic and metabolic similarity between bacteria increases the probability of antagonism [48].

Experimental

Strains, media and chemicals. All strains used in this study are listed in Table S1 (Supporting Information File 1). For routine growth, bacterial cells were cultured in tryptic soy broth (TSB, CASO Broth, Sigma-Aldrich) containing 17 g l^{-1} casein peptone, 3 g l^{-1} soy peptone, 5 g l^{-1} sodium chloride, 2.5 g l^{-1} dipotassium hydrogen phosphate and 2.5 g l^{-1} glucose.

Semi-synthetic mock community assay. Semi-synthetic soil communities were obtained from soil of sampling site P5 (55.788800, 12.558300) [43,49]. 1 g soil was mixed in a 1:9 ratio with 0.9 % saline solution, vortexed on a rotary shaker for 15 min and allowed to sediment for 2 min. Four independent communities were established by inoculating 10-times diluted TSB (0.1x TSB) with 1 % soil suspension taken from the middle part of the liquid phase, followed by incubation at 21-23°C and 250 rpm for 12 h. Simultaneously, pre-grown *B. subtilis* P5_B1 WT and its NRP mutant derivatives were inoculated in 0.1x TSB and incubated parallel using the same conditions. After 12 h pre-cultivation, 3 ml aliquots of the soil suspension were transferred into six glass tubes. One tube was left untreated and functioned as control, the remaining five were supplemented with respective *B. subtilis* strains by adding 10 % of the final volume. The cultures were incubated at 21-23°C and 250 rpm for 48 h. DNA was extracted from two soil aliquots, the pre-cultured soil suspensions and each assay supplemented with the respective *B. subtilis* strains as well as untreated control assays after 48 h co-cultivation.

DNA extraction. Environmental and semi-synthetic community genomic DNA were extracted from either 250 mg soil or 250 µl bacterial culture, respectively, with DNeasy PowerSoil Pro

Kit (QIAGEN) following the manufacturer's instructions.

Amplification of 16S rRNA hypervariable regions V3-V4. The V3-V4 region of the 16S rRNA gene was PCR amplified from the extracted DNA samples using Fw_V3V4 (5'-CCTACGGGNGGCWGCAG-3') and Rv_V3V4 (5'-GACTACHVGGGTATCTAATCC-3') primers that were tagged with short barcodes with a length of eight nucleotides, listed in Table S2 (Supporting Information File 1). The PCR reactions contained 10.6 µl DNAse free water, 12.5 µl TEMPase Hot Start 2x Master Mix, 0.8 µl of each primer (10 µM) and 0.3 µl of 50 ng/µl DNA template. The PCR was performed using the conditions of 95°C for 15 min followed by 30 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, and finally, 72°C for 5 min. All V3-V4 amplicons were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and pooled in equimolar ratios. The amplicon pool was submitted to Novogene Europe Company Limited (United Kingdom) for high-throughput sequencing on an Illumina NovaSeq 6000 platform with 2 million reads (2x 250 bp paired-end reads). Raw sequence data is available at NCBI: PRJNA658074.

Sequencing data pre-processing. The multiplexed sequencing data was imported into the QIIME 2 pipeline (version 2020.6) [50,51]. The paired-end sequences were demultiplexed with the QIIME 2 plugin cutadapt [52]. To reduce random matches, the minimum overlap of partial matches between the read and the barcode sequence was set to 5 nucleotides. The QIIME 2 implementation DADA2 was used to denoise and merge paired end reads [53]. In total, 362,475 reads were assigned to the respective samples with an average of 12,083 reads per sample (range: 751 to 34,802; Supporting Information File 1, Table S3). The amplicon sequence variants (ASVs) were clustered at 99 % identity into OTU using VSEARCH [54]. The

16S rRNA reference sequences with a 99 % identity criterion obtained from the SILVA database release 132 were trimmed to the V3-V4 region, bound by the primer pair used for amplification and the product length was limited to 200-500 nucleotides [55]. Taxonomy was assigned to the sequences in the feature table generated by DADA2 by using the VSEARCH-based consensus taxonomy classifier [54]. A tree for phylogenetic diversity analyses was generated with FastTree 2 from the representative-sequences [56–58].

Relative species abundance and phylogenetic diversity analyses. QIIME 2 artifacts were imported into the R software (4.0.2) with the R package qiime2R and further analyses were conducted in the R package phyloseq [59–61]. Differences in abundances of the most abundant genera in the control communities, in the communities supplemented with *B. subtilis* WT as well as in the communities supplemented with *B. subtilis sfp* were investigated by calculating the abundance ratios of the different treated communities for each replicate. If species were not detected in some of the replicates, 0 values were replaced with the lowest detected value of the genus to avoid infinite values or 0 values in the ratio calculations. Diversity analysis of the *B. subtilis*-treated and untreated samples were performed with OTU counts multiplied with factor 100,000 and transformed into integer proportions. Alpha diversity was estimated with the Shannon diversity index in the R package phyloseq [61]. Beta-diversity was determined by dissimilarities among the samples with the Bray-Curtis distance and visualized in a nonmetric multidimensional scaling (nMDS) with the R package vegan [62]. All graphical visualizations were realized with ggplot2 [63].

Statistical analysis. Statistical significance was determined with the square roots of the tested values. Normality and equality of variances were tested with Shapiro-Wilk normality test and

Levene's test, respectively. If one of the tests was rejected the non-parametric Kruskal-Wallis rank sum test was performed instead. The statistical significance of pairs was determined with the Welch two sample t-test and the differences among groups > 2 was determined with one-way analysis of variance (ANOVA) test and Tukey's HSD test. Statistical significance was determined with an alpha level < 0.05.

Growth monitoring of *L. fusiformis* supplemented with *B. subtilis* spent media and pure surfactin. Spent media of *B. subtilis* strains were harvested from cultures grown in TSB medium at 37°C and 250 rpm for 48 h immediately before the growth experiments. Cultures were adjusted to OD_{600} 3.0 and centrifuged for 4 min at 5,000× g. Subsequently, the supernatants were passed through 0.22 µm filters and stored at 4°C. The growth experiments were performed in 96-well microplates. The wells of the first column were filled with 30 µl 10x TSB, 30 µl *L. fusiformis* culture adjusted to OD_{600} 0.1 in 1x TSB and 240 µl of the appropriate *B. subtilis* spent medium or water (untreated control). 100 µl *L. fusiformis* culture adjusted to OD_{600} 0.01 in 1x TSB was added to the wells of the remaining columns. A 1.5-fold serial dilution of the spent media was performed column-by-column. A surfactin stock solution was prepared by dissolving 10 mg of surfactin (Sigma-Aldrich) in 1 ml methanol (MeOH). The wells of the first column were filled with 170 µl 1x TSB, 20 µl *L. fusiformis* culture adjusted to OD_{600} 0.1 in 1x TSB, and 10 µl surfactin, 10 µl MeOH (solvent control) or 10 µl 1x TSB (untreated control). To the wells of the remaining columns, 100 µl *L. fusiformis* culture was added adjusted to OD_{600} 0.01 in 1x TSB. A 2-fold serial dilution of surfactin or MeOH was performed column-by-column. In both assays the growth of *L. fusiformis* was monitored in a microplate reader (BioTek Synergy HTX Multi-Mode Microplate Reader). The microplates were incubated at 30°C with continuous shaking (548 cpm, 2 mm) and the OD_{600} was

measured in 15 min-intervals over a period of 24 h. All graphical visualizations were prepared using ggplot2 [63].

Supporting Information

Supporting information features the bacterial strains used in this study (Table S1), the 16S rRNA V3-V4 primer list (Table S2), the number of sequencing reads per sample (Table S3) and supporting figures (Figure S1-S4).

Supporting Information File 1:

File Name: SupportingTables.pdf

File Format: PDF

Title: Supporting tables

Supporting Information File 2:

File Name: Figure S1.png

File Format: PNG

Title: Abundance ratios for each genus in the *sfp*-treated community compared to the WT-treated community.

Detailed description: Abundance ratios for each genus and replicate (points) in the *sfp*-treated community compared to the WT-treated community. Statistical significance is defined as $P \leq 0.05$ (*) and $P \leq 0.001$ (***).

Supporting Information File 3:

File Name: Figure S2.png

File Format: PNG

Title: Abundance of *Viridibacillus* genus in each mock community.

Detailed description: Abundance of *Viridibacillus* genus in each mock community. Points represent the abundance in each replicate. Treatments with the same letter are not significantly different.

Supporting Information File 4:

File Name: Figure S3.png

File Format: PNG

Title: Different levels of aggregation of *L. fusiformis* M5.

Detailed description: Different levels of aggregation of *L. fusiformis* M5 after exposure to spent medium of *B. subtilis* WT and *sfp* or without treatment. Scale bars indicate 3 mm.

Supporting Information File 5:

File Name: Figure S4.png

File Format: PNG

Title: Growth curves of *L. fusiformis* M5 exposed to the solvent MeOH.

Detailed description: Growth curves of *L. fusiformis* M5 exposed to different concentrations of the solvent MeOH and without treatment (control). Error bars represent the standard error. N = 6 (control assay), N = 2 (MeOH-treated assays)

Acknowledgement

This project was supported by the Danish National Research Foundation (DNRF137) for the Center for Microbial Secondary Metabolites (CeMiSt).

The authors thank the suggestions of Lone Gram and the CeMiSt consortium members on the project.

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