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Harnessing Intercellular Signals to Engineer the Soil Microbiome

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REVIEW

Harnessing Intercellular Signals to Engineer the Soil Microbiome

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Plant and soil microbiomes consist of diverse communities of organisms from across kingdoms and can profoundly affect plant growth and health. Natural product-based intercellular signals govern important interactions between microbiome members that ultimately regulate their beneficial or harmful impacts on the plant. Exploiting these evolved signalling circuits to engineer microbiomes towards beneficial interactions with crops is an attractive goal. There are few reports thus far of engineering the intercellular signalling of microbiomes, but this article argues that it represents a tremendous opportunity for advancing the field of microbiome engineering. This could be achieved through the selection of synergistic consortia in combination with genetic engineering of signal pathways to realise an optimised microbiome.

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1 Introduction

Microbiomes engage in key interactions with associated multicellular eukaryotes, from influencing human gut health to interacting synergistically with fungus-farming ants[1,2]. The soil microbiome, the community of microorganisms (bacteria, fungi, protists and archaea) in the soil environment, is crucial to plant health and growth[3]. Modern DNA sequencing technologies have readily allowed for the identification of the constituents of the soil microbiome and the genes within those species, including those members that classically have been difficult to study as they have to date proved unculturable in the lab. Microbiome composition varies with external factors such as pH, temperature, water levels and agriculture methods; for instance, increases in the frequency of *Streptomyces* species were observed with longer crop rotation intervals[4–8]. Plants can also influence their associated microbiomes, including through root exudates such as jasmonic acid (**1**) or triterpenes[9–13]. Plant-mediated shifts in composition can occur rapidly during the lifecycle of the plant; for example, *Arabidopsis* in late growth stages enriches nitrogen-fixing bacteria[14]. The soil microbiome in agricultural fields commonly includes plant pathogens such as the bacterium *Pseudomonas syringae* or the fungus *Claviceps purpurea*[15,16], but it can also provide disease suppression through ubiquitous genera such as *Bacillus*, *Pseudomonas* and *Streptomyces*[17–21]. Numerous plant growth promoting bacteria (PGPB) have been discovered[22], that can improve growth of

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crops such as rice[23], including in the presence of soil contaminants such as copper[24], through diverse mechanisms.

Given the major impact of the soil microbiome on crops, the development of enhanced soil microbiomes for agricultural use is an attractive goal. While traditional approaches have focused on crop rotation or the use of organic amendments, including green manures, more recent work has focused on bio-inoculation and host mediated-evolution[25–30]. To optimise a microbiome for its associated plant, it is necessary to add or facilitate organisms that carry genes that encode plant-beneficial functions. However, such a strategy has obvious limitations: for example, in the bacterial genus *Streptomyces*, which is particularly important for plant health, secondary metabolite biosynthetic gene clusters are often silent, i.e. not expressed under laboratory conditions[31–34]. In a case like this, it is not sufficient for the beneficial genes to be present in the microbiome gene pool, but they also have to be expressed. That is to say, the correct signal or stimulus needs to be present to unlock their beneficial phenotype. This can be most directly achieved by manipulation of microbiome intercellular signalling; this goal, therefore, represents an exciting and relatively unexplored avenue towards the enhancement of soil microbiomes[35]. However, to realise this goal and effectively reverse-engineer the microbiome, we first need to consider our current knowledge of signalling within microbiomes.

1.1 Defining a signal

In the broad sense, a molecule produced by an organism that elicits a reaction in another organism is considered a *signal*. However, this usage has often been considered too unspecific, and alternative definitions have been variously proposed. For example, according to the more narrow criteria of Diggle and colleagues[36], which we apply here, only molecules involved in a system that has evolved due to a fitness benefit to both sender and receiver are considered as *signals* in the strict sense. In contrast, where an excreted molecule does not impart a fitness benefit to the sender, but only to the receiver, it is considered a *cue*. Systems that have evolved so that the secreted molecule induces a response in a receiver without associated fitness benefit are considered *coercive*.

Winzer, Hardie & Williams propose alternative criteria, which rely on functional rather than evolutionary characteristics, to define when a natural product should be considered a cell-to-cell signal molecule[37]. Production of the signal must occur at specific growth stages or environmental conditions. It must accumulate extracellularly, be recognised by a specific receptor, and generate a concerted response at a threshold concentration. The response must extend beyond metabolism or detoxification of the signal.

A case where the different definitions of signals become relevant are sub-inhibitory concentrations of antibiotics (SICA). According to the functional criteria of Winzer and colleagues, SICA could be considered signals[38], as they can elicit responses beyond resistance, such as altering nutrient use in the receiver cell[39]. However, communication via SICA effecting changes to nutrient use are not likely to confer a fitness benefit to the sender; thus, according to the evolutionary definition of Diggle and colleagues, SICA would be considered cues, rather than signals. But in an alternative scenario, where sensing of an antibiotic promotes co-operative biofilm formation, it confers a fitness benefit to the sender as well and can be considered a signal in the strict sense. Thus, dependent on the response elicited, antibiotics can be cues or signals by this definition[40].

2 Natural product signals in the soil microbiome

Soil microbiome constituents use a variety of intercellular signals (**1-11**; Figure 1) and cues to regulate natural product (**12-23**; Figure 2) biosynthesis, and to mediate interactions with the surrounding plants and other microbial species. These range from PGPB-produced auxins to antibiotics at sub-inhibitory concentrations. Understanding of the enzymatic pathways responsible for signal transmission, reception and response is an essential prerequisite to their use in engineering a signal-optimised microbiome towards plant health.

2.1 Quorum sensing and inhibition

A well-studied example of intercellular microbiome signalling is quorum sensing (QS) in diverse bacterial populations. Signalling interactions among *Pseudomonas*, are of particular interest as this genus includes both PGPBs and notorious plant pathogens (e.g., *P. syringae*), as well as a number of opportunistic pathogens (including the human pathogen *P. aeruginosa*). All of these use acyl-homoserine lactone (AHL) QS to regulate virulence factors such as pyocyanin (**12**)[41,42]. Canonically, QS includes a LuxI-type AHL synthase and LuxR transcriptional regulator that detects the signal; however, organisms containing only LuxR also exist (without a corresponding LuxI AHL synthase) that can sense other signals such as photopyrones (**2**)[43]. As *Pseudomonas* species are influential to plant health, and QS perhaps the most studied class of signalling, QS is an auspicious choice for the genetic engineering of intercellular signalling.

Importantly, in nature, QS does not simply occur between members of one species, but rather can be influenced by other microbes and plants, via crosstalk and eavesdropping interactions. For instance, *Streptomyces* can produce quorum sensing inhibitory (QSI) compounds that interrupt *P. aeruginosa* QS regulation and pathogenesis[44]. Organisms can also produce enzymes that degrade quorum sensing signals (of their own or other species), in a processes called quorum quenching (QQ)[45]. *Agrobacterium fabrum* (formerly known as *Agrobacterium tumefaciens*) produces QQ enzymes that degrade the bacteria's own QS AHL, as part of a regulatory system for conjugative transfer of the tumour-inducing plasmid[46]. In generating a signal-optimised microbiome, QSI and QQ could both be used to inhibit QS systems regulating plant pathogen virulence factors. Indeed, this would mimic an interaction that has evolved in some soil microbiomes in nature, where the PGPB *Pseudomonas segetis* P6 was observed to degrade a broad range of AHLs and consequently confer protection from pathogens such as *Pseudomonas syringae* pv tomato[47].

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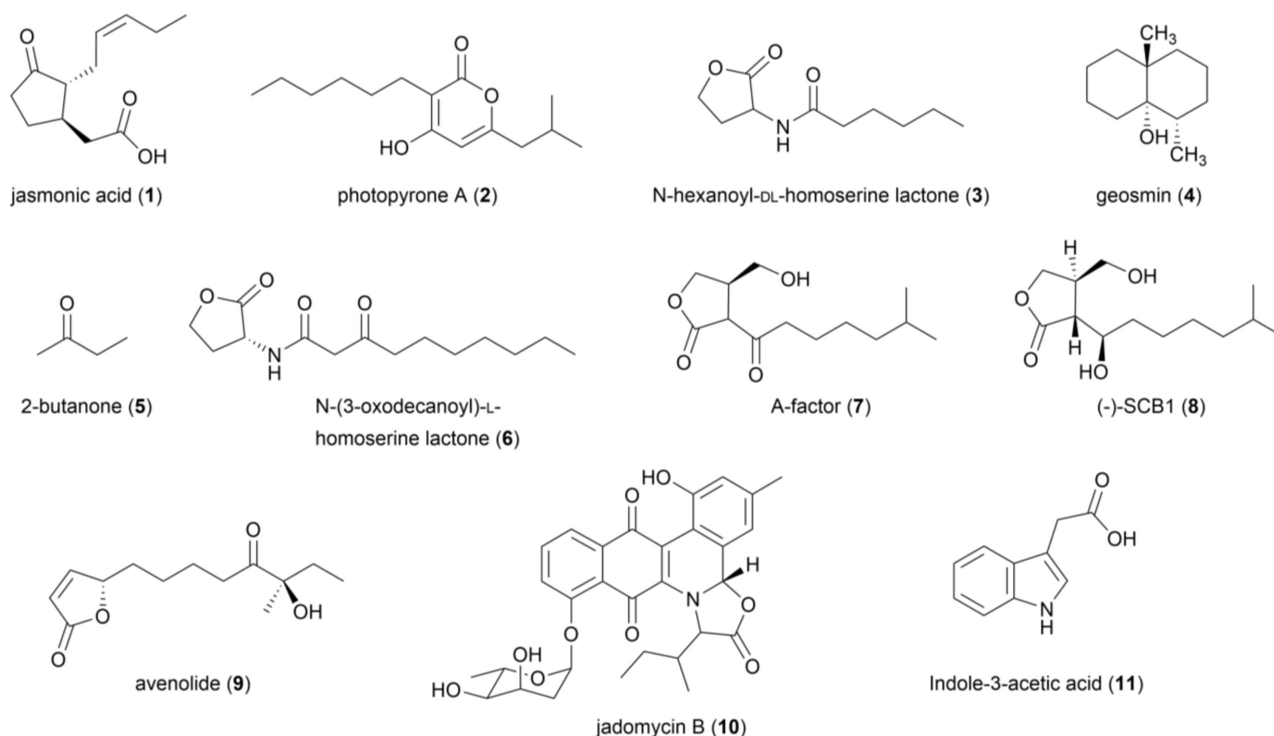

Figure 1 – Examples of natural product signalling molecules in the soil

Whilst often associated with pathogenesis, bacterial QS can also be directly beneficial to plants which can detect bacterial AHLs[48]. In *Arabidopsis*, introduction of *N*-hexanoyl-DL-homoserine-lactone (**3**) induced changes in the transcriptome and promoted root growth, whereas *N*-decanoyl-DL-homoserine-lactone decreased root growth[49]. Bacterial AHLs can both promote and downregulate sporulation of moss in a concentration-dependent manner[50]. Therefore, when considering optimising QS to benefit the plant in the microbiome, it is not simply a matter of inhibiting or quenching all signals.

2.2 Cross-kingdom signalling

Plant eavesdropping on microbial QS is an example of cross-kingdom signalling, which could have powerful effects when engineered carefully. There are other known cases of cross-kingdom interactions with important effects on plant health; for instance, bacterial LuxR-type regulators have evolved to sense plant exudates, such as OryR in the pathogen *Xanthomonas oryzae* pv. *oryzae*, which can sense an uncharacterised molecule secreted by rice plants, inducing expression of genes related to motility and virulence[51]. Cross-kingdom signalling has also been observed from the yeast *Saccharomyces cerevisiae* to the bacterium *Streptomyces venezuelae*, where trimethylamine induced unusual horizontal hyphal growth, independent of the canonical *Streptomyces* developmental regulators (*bld* and *whi*)[52,53]. These examples demonstrate that microbiome signal engineering needs to be considered within the context of the whole microbial community and associated plants. It is conceivable that a pairwise signalling interaction characterised between two organisms in a laboratory setting could have unexpected effects on other members of a diverse microbiome.

Cross-kingdom signalling can also be directed towards insects; virtually all *Streptomyces* strains produce geosmin (**4**), which attracts springtails[54]. Geosmin biosynthesis is under the regulation of sporulation-specific transcription factors, suggesting that it may have evolved to promote the spread of spores in the soil via the insect. Such signals could be used to modulate insect populations, as demonstrated by the significant differences observed in aphid numbers per ragwort plant when grown in soils preconditioned with different plants; an effect postulated to be mediated by soil fungal communities[55]. Soil microbiomes from different crop soils can affect the behaviour of insects, decreasing larval feeding on *Arabidopsis thaliana*[56]. Signals could also be used to recruit beneficial insects, as in the case of ladybugs being attracted by synthetic 2-butanone (**5**)[57]. Synthetic biology allows us to develop microbial *in vivo* biosynthetic pathways to produce such signals, such as in *E. coli* engineered for 2-butanone production[58]. Signalling to insects, whether to attract, repel or modulate their behaviour, provides an important avenue through which microbiome engineering could benefit crop health.

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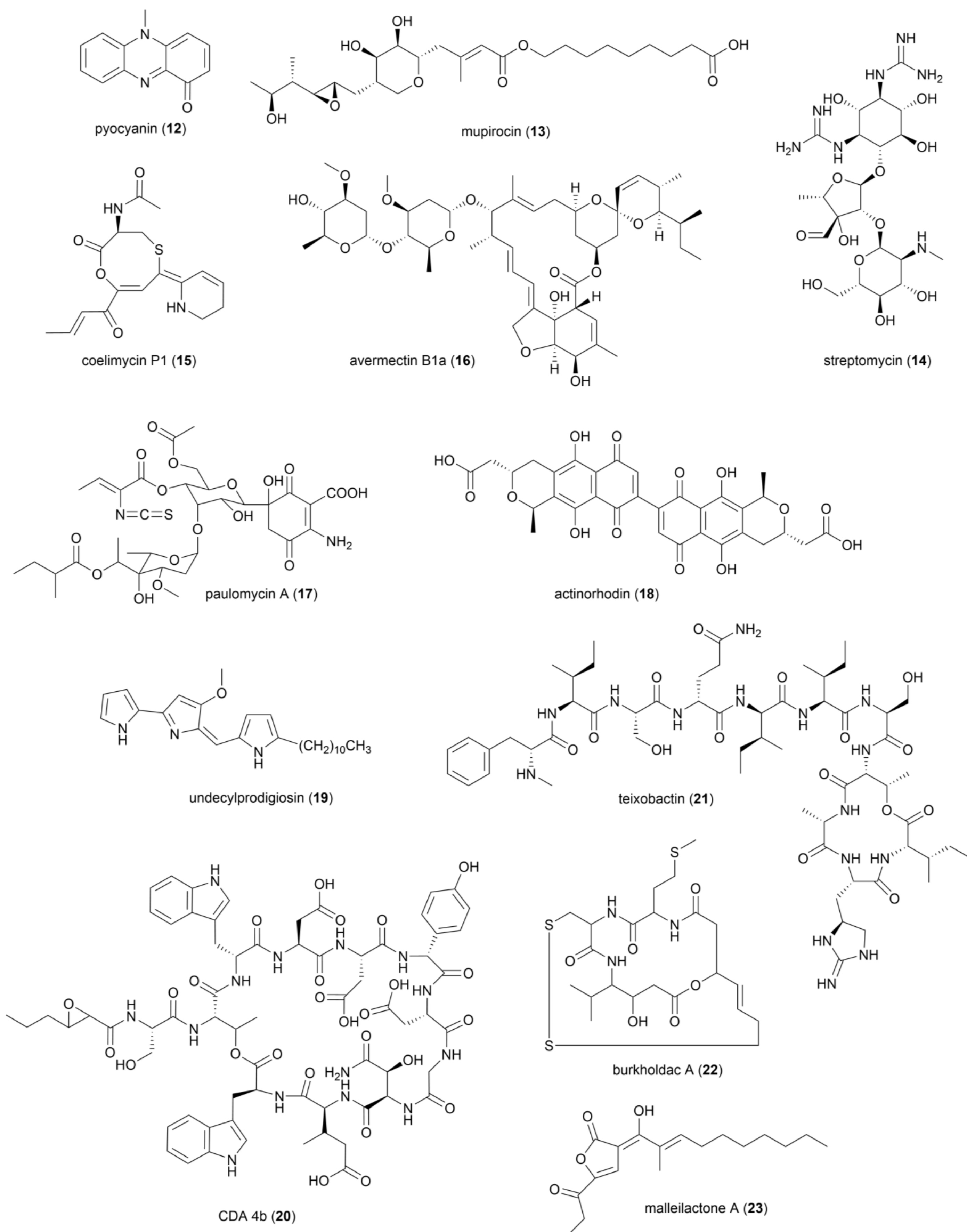

Figure 2 - Natural Products discussed in this review

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2.3 Regulation of natural product biosynthesis

Soil microbiomes can be disease-suppressive through activities such as the production of antibiotics by their constituent bacteria, which is typically regulated by intercellular signals. QS systems have evolved to regulate natural product biosynthesis, such as for mupirocin (**13**) production in *Pseudomonas fluorescens.* The biosynthetic gene cluster (BGC) for mupirocin includes *mupI*, encoding an N-Acyl homoserine lactone synthase; the addition of exogenous N-(3-Oxodecanoyl)-L-homoserine lactone (**6**) signal was observed to restore antibiotic production in a *mupI* knockout strain, demonstrating this natural product signal is required for mupirocin biosynthesis[59]. However, addition of the lactone to the WT producer did not increase titre, nor stimulate early antibiotic production, indicating the multi-level regulation of such natural product pathways[60].

The prolific secondary metabolite-producing genus *Streptomyces* uses γ-butyrolactones (GBL) and γ-butenolides signals to regulate secondary metabolite production, such as regulation of streptomycin (**14**) biosynthesis by A-factor (**7**) in *Streptomyces griseus*[61–64]. Similarly, *Streptomyces coelicolor* butyrolactones (SCBs, **8**) act as a diffusible signal, able to relieve ScbR repression at promoters such as for *cpkO*, which encodes an activator for the coelimycin (**15**) BGC[65–67]. In *Streptomyces avermitilis*, the cognate GBL avenolide (**9**) induces production of the insecticide avermectin (**16**). No increase in production of avermectin was observed with SCB1, however, providing evidence of the specificity of these signals[62]. As well as being of major interest in drug discovery, understanding and engineering this intercellular regulation to potentially switch on silent biosynthetic gene clusters encoding metabolites that benefit the plant should be considered as a promising strategy towards generating a signal-optimised microbiome[35].

Where typically a single bacterial species will both produce and detect the signals regulating secondary metabolism, there is also evidence of cross-strain signalling. Genome analysis of *Streptomyces albidoflavus* J1074 revealed the presence of a predicted GBL receptor but no biosynthesis genes, and intriguingly heterologously introducing *S. coelicolor* GBLs induced paulomycin (**17**) biosynthesis[68]. This again highlights the need to consider multiple microbiome members when engineering signalling.

2.4 *Streptomyces coelicolor* – a case study of soil microbial signalling

As alluded to in the previous section, the regulation of natural product biosynthesis is not typically as simple as inducing production in response to a single signal. Rather, each signal provides one input into a complex regulatory network that is not always well understood. The model soil bacterium *S. coelicolor* provides an excellent exemplar given the importance of its genus for natural product biosynthesis and the extensive previous studies into its secondary metabolite regulatory networks.

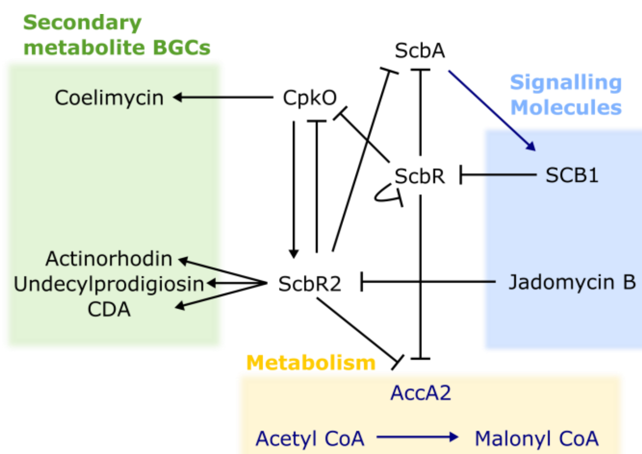

Figure 3 - Interaction of signals with selected transcription factors regulating natural product biosynthesis in *S. coelicolor*. Black arrows represent activation and repression, blue arrows chemical reaction/biosynthesis[71-73]. See text for details.

The complexity of the network function is demonstrated by the observation that deletion of *scbA*, without which SCB signalling molecules are not biosynthesised, increases the production of actinorhodin (**18**) and undecylprodigiosin (**19**)[65], which are not directly under SCB regulation. Later ChIP-seq, transcriptomic and proteomic studies revealed the network responsible for this phenotype (summarised in Figure 3), and the pleiotropic effects of these signals[69–71]: deletion of *scbA* abolishes biosynthesis of the SCB signals, which are therefore not available to bind to transcription factor ScbR. Transcription of *scbR* was observed to be diminished in the Δ*scbA* mutant, as ScbR represses its own promoter[65]. ScbR represses production of another transcription factor CpkO, and therefore deletion of *scbA* should increase *cpkO* expression. CpkO activates expression of the pseudo-GBL receptor ScbR2, which activates transcription factors regulating actinorhodin, undecylprodigiosin and calcium-dependent antibiotic (CDA, **20**) biosynthesis. ScbR2 does not bind SCBs, but instead responds to antibiotic signals such as the endogenous actinorhodin and undecylprodigiosin, as well as jadomycin B (**10**) produced by *Streptomyces venezuelae*[72]. ScbR and ScbR2 interact with the regulation of glucose catabolism, for example repressing production of acetyl-CoA carboxylase AccA2, controlling the flux from acyl-CoA to malonyl-CoA precursor for these polyketide natural products[70]. The regulatory network is not limited to transcription factors; for example, the global regulator *bldA* encodes a tRNA^{LEU} for the rare codon UUA, allowing for translational control of RedZ in undecylprodigiosin biosynthesis[73].

These examples from *S. coelicolor* are far from exhaustive, but they indicate the complexity of the regulatory networks that signalling molecules perturb. Understanding of these networks and the wider indirect effects of signals is key to successfully engineering signalling within a microbiome. This also emphasises the importance of –omics techniques in studying these effects, as discussed in the following sections.

2.5 Uncovering signalling in uncultured bacteria

GBL circuits in *Streptomyces* and QS in *Pseudomonas* species are well-studied signalling systems, where many signalling molecules and cues have been characterised, together with the molecular mechanisms cells use to respond to them. The many studies that have contributed to this knowledge have often relied on the culturability of the signalling partners in the lab. However, it is estimated as little as <1% of bacteria are culturable under standard laboratory conditions, limiting the possibilities for characterising signalling in this manner[74]. Furthermore, under laboratory conditions, microbes might not produce and respond to signals as they would in a natural soil ecosystem. The experimental parameters are complex; studying a given signalling pathway in the laboratory may require certain media, temperature, pH or combinations of organisms. It may be difficult to identify a metabolically inactive signaller or responder from a natural system, but this is essential before being able to reproduce the signalling in the lab. This means that potentially most bacterial signals and their effects are yet to be investigated. Expanding our understanding of these signals is important to achieving the goal of a signal-optimised microbiome that benefits crops.

One way of overcoming the culturability barrier is to develop technology to dramatically increase the range of culturable bacteria, such as the isolation chip (iChip) technology, which facilitated discovery of a promising new antibiotic, teixobactin (**21**), from a previously inaccessible microbe[75,76]. Despite these efforts, a large proportion of the microbiome likely remains uncultured for the foreseeable future. An alternative route of access is provided by *in situ* methodologies. Metagenomic analyses can reveal the gene pool of uncultured microbial communities, and potential signalling interactions can be predicted through genetic homology to known systems. However, this intrinsically limits the novelty of discoveries. Metatranscriptomics have been used to gain insight into the gene expression of the microbiome in response to environmental stimuli such as soil contamination and global warming[77–79]. It also allows for the investigation of the gene expression patterns underlying signal biosynthesis, as demonstrated in phytoplankton-associated bacteria with indole-3-acetic acid (**11**) signalling[80], and could be used to monitor the wider effect of introducing a signal-optimised consortium.

2.6 Studying the effects of signals

Indeed, in general, an alternative to investigating the signals themselves is to probe cells responses instead, looking at changes in transcription, metabolism, or phenotype in response to potential signals. Introduction of reporter genes into two silent gene clusters for burkholdac A (**22**) and malleilactone A (**23**) in *Burkholderia thailandensis* allowed for the high-throughput identification of elicitors, potential signalling molecules, from a library of 640 compounds, an exciting proof of concept[81]. This information could be used, together with genetic engineering of the biosynthesis of these elicitors, to develop orthogonal signalling circuits that can maintain and regulate novel microbiome components independently of the native soil microbiome.

With the maturity of RNA-seq, transcriptomics can yield insight into genome-wide expression effects of a signal. For example, this approach has been used to elucidate the *Pseudomonas syringae* transcriptome response to the plant immune system[82]. Concurrent use of multiple molecular profiling technologies represents a promising avenue to comprehensively characterise signalling in a microbiome; to effectively bring these complex datasets together to predict the emergent properties of a signalling network from genome to transcriptome to metabolome and phenotype will require the development of computational models[83]. Models have been developed for understanding signalling circuits, such as γ-butyrolactone signalling in *S. coelicolor*[66], or to predict the metabolic interactions within an entire multi-species community, as demonstrated with the experimentally-validated prediction of the equilibrium of a three-species consortium with COMETS[84]. As we expand our understanding of signalling in the soil by diverse complementary methodologies, we increase our possibilities for its reverse-engineering. We are better able to predict how our perturbations will affect other organisms in the microbiome and therefore how to design signalling circuits in the context of a microbial consortium to benefit plants.

3 Manipulation of soil microbiomes

It is important to consider the avenues available for achieving a signal-optimised microbiome with tangible benefits to crops in practice. Options include use of soil additives, mobile genetic elements or bio-inoculation of an optimised consortium, each with their advantages and disadvantages, and with the possibility of concurrent use.

3.1 Chemical and enzyme additives to soil

The composition of the crop microbiome is heavily influenced by agricultural practices[85], including the use of fertilisers, pesticides, and organic amendments, which affect the microbiome in a soil-specific manner[86]. For instance, addition of biochar to Chinese ginseng soil enriched populations of *Bacillus*[87], whereas in rice soils ^{13}C-labelled biochar was associated with preferential metabolism by Gram negative species, compared with addition of straw and rice root[88]. Carbon amendment through the addition of compounds such as fructose and glucose was observed to alter bacterial community composition and enrich *Streptomyces* antagonistic phenotypes[89,90]. These factors are important when considering the practical application of an optimised microbiome to crop soils; it might be that certain fertilisation treatments and agricultural practices promote the perseverance of beneficial consortia.

The direct addition of enzymes to soil could also be considered for degrading signals. Lactonase enzymes that specifically degrade AHLs have been introduced to a bioreactor within silica capsules, resulting in decreased *Pseudomonas* biofilm formation[91]. However, it would be challenging to

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achieve this on a large scale, to protect the enzymes in the soil environment and to deliver them precisely to the locale required for function. Furthermore, the general degradation of AHLs is not desirable, as these can regulate plant-beneficial effects and AHLs would have a significant role in intercellular signalling in our model microbiome. However, the concept of adding enzymes that affect soil signalling in a contemporary manner could be used to control signalling and therefore phenotypes. For example, a lactonase could be added that degrades a specific AHL, the absence of which has been designed to promote phosphate solubilising gene pathways within the designed rhizosphere. This could allow *in situ* control of the phenotype, for instance allowing us to increase phosphate solubilisation by desirable bacteria[92]. The direct addition of the enzymes to soil would likely be short-lived and may prove impractical; an alternative approach could be to inoculate with a microbe that produces and secretes the enzyme instead. These proposed exogenous control systems could supplement a genetically-engineered microbiome towards plant benefit.

3.2 Genetic engineering of the microbiome *in situ*

The genetic engineering of the microbiome *in situ* has so far been of particular interest in the study of animal-associated microbiomes[93]. It is achieved through the introduction of mobile genetic elements: plasmids and bacteriophages. In the mouse gut, conjugative plasmids in combination with the Himar transposon were successful in transmitting test reporter genes (GFP and carbenicillin resistance) through the microbiome[94]. A prudent choice of plasmid for soil microbiomes could be the broad-host range RP4, which is self-transmissible to both Gram positive and negative strains and also encodes a toxin–antitoxin-based addiction system and DNA partition mechanisms to prevent plasmid loss. Inoculation of vegetable field soil with *Pseudomonas putida* carrying an RP4-derivative demonstrated the ability of the plasmid to transfer to the existing soil microbiome and persist over a 75-day period[95]. However, such approaches do not allow for fine control; it is impossible to predict which bacteria would receive the plasmid, and there is potential for non-target effects. Indeed, in a natural cautionary tale, adhesion systems aiding plant growth promotion in *Pseudomonas* may have undergone horizontal gene transfer to *Erwinia carotovora*, within which they contribute to plant virulence[96]. An advantage of engineering *in situ* is that the existing microbiome has already evolved for its niche and can therefore be expected to persist. However, given the inherent lack of control and the significant ethical and regulatory boundaries to *in situ* genetic engineering, bio-inoculation with engineered consortia is a more attractive option in soil.

3.3 Bio-inoculation of soil with beneficial bacteria

The use of bacterial and/or fungal bio-inoculants to benefit plants is well-established, with diverse studies demonstrating plant growth promotion and pathogen antagonism[97–101]. In theory, the inoculation of crop soil with PGPB or disease-suppressive bacteria can provide an immediate means to benefit agriculture. In a simplistic example, one could identify a new *Streptomyces* strain that in lab cultures produces an antibiotic effective against plant pathogens and expect inoculation of crop soil to provide pathogen suppression. However, the inoculant must invade and persist in the natural microbiome[102], as has been demonstrated in the mammalian gut with the colonisation of genetically engineered strains[103,104], and it must produce or receive the relevant intercellular signals to direct the production of the antibiotic. Indeed, even in greenhouse experiments, persistence can be a problem; e.g., the population of two PGPB strains was observed to drop by 95% and 99% between 2 and 5 days post inoculation[105]. A potential solution to this is to deliver the inoculum by a different means. In Chinese kale soil, colonisation and plant growth promotion of *Ensifer fredii* was achieved when immobilised in agar, where liquid culture inocula failed[106]. However, a solution to persistence issues might be to apply a consortium that acts synergistically, which also furthers the possible beneficial phenotypes mediated by signalling that can be realised. In an example of co-operation, co-inoculation of *Paenibacillus mucilaginosus* and *Sinorhizobium meliloti* mediated greater growth promotion of alfalfa than either inoculant individually[107]. The survival of introduced *Pseudomonas* communities increased with increased microbial diversity of the inoculum, also corresponding with pathogen suppression[108]. In the field, inoculation of degraded arable land with nearby biodiverse heathland and grassland soil effects a profound increase in plant species coverage over a period of six years[109]. This demonstrates that there is good scope for the application of an engineered microbiome to a real-world field to deliver lasting benefits. Indeed the engineering of microbiomes was the focus of the most recent Engineering Biology Research Consortium roadmap, that establishes the diverse anticipated outcomes over the next 20 years, from engineering spatial properties to distributing the burden of compound biosynthesis[110].

4 Building an optimised consortium

4.1 Selection of consortium members

A key step in curating a signal-optimised microbiome is choosing its constituents[35]. Optimisation of signalling need not be restricted to genetic engineering approaches; combinations of strains that natively exchange signals that support plant-beneficial phenotypes could underpin the selection of microbiome constituents. One should also consider that bacteria can promote the growth of other strains; for example, the presence of *Streptomyces pactum* increases the population of PGBP *Pseudomonas koreensis* GS in the rhizosphere[111]. Microbiome constituent selection is key: in nature, members of the microbiome have evolved in complex communities, undergoing diverse species interactions within and across kingdoms. The effects of cross-kingdom species interactions on functional capacities is evidenced by significantly greater inhibition between sympatric, co-evolved *Fusarium* and *Streptomyces* populations than allopatrically evolved strains[112].

533 When a 185-member synthetic bacterial community was applied to *Arabidopsis* seedlings, interference with auxin signalling mediated by the auxin-degradation operon conserved within the genus *Variovorax* was observed as being key for normal root development[113]. Typing of 16S rRNA has been used in studies to determine the core bacterial taxa present in geographically distinct replicates of crop-associated rhizospheres[114–116]. While the number of core taxa reported in these experiments varies, they support the idea that there are core phyla, such as Proteobacteria, almost ubiquitously present across soils. This suggests a substantial level of robustness and persistence in these taxa, and it may be sensible to develop candidate strains for engineering from within this stable core.

4.2 Modern technologies for the genetic engineering of soil bacteria

The real potential power of signalling can be unlocked through the engineering of the genes and pathways encoding and responding to these signals. We have more capability to genetically engineer diverse bacteria than ever before, particularly with the maturation of CRISPR methodologies for bacterial genome engineering. In the prolific antibiotic-producing genus *Streptomyces*, for example, CRISPR-Cas9 plasmids are available for precise genetic engineering mediated by specific DNA double strand breaks, alongside multiplex CRISPRi and base editing vectors[117–119]. Whilst there is no guarantee that these work in all *Streptomyces* strains, CRISPR-Cas9 plasmids are available with differing constitutive and inducible regulation of *cas9*, which allows for tuning to mediate any Cas9 toxicity issues[120–123]. These systems have supplemented existing engineering options, such as phage serine integrase mediated insertions, suicide plasmid-based homologous recombination and replicative plasmid gene expression[124]. There is a plethora of molecular biology cloning methods for the efficient construction of these mutagenesis plasmids from Golden Gate to Gibson Assembly[125,126].

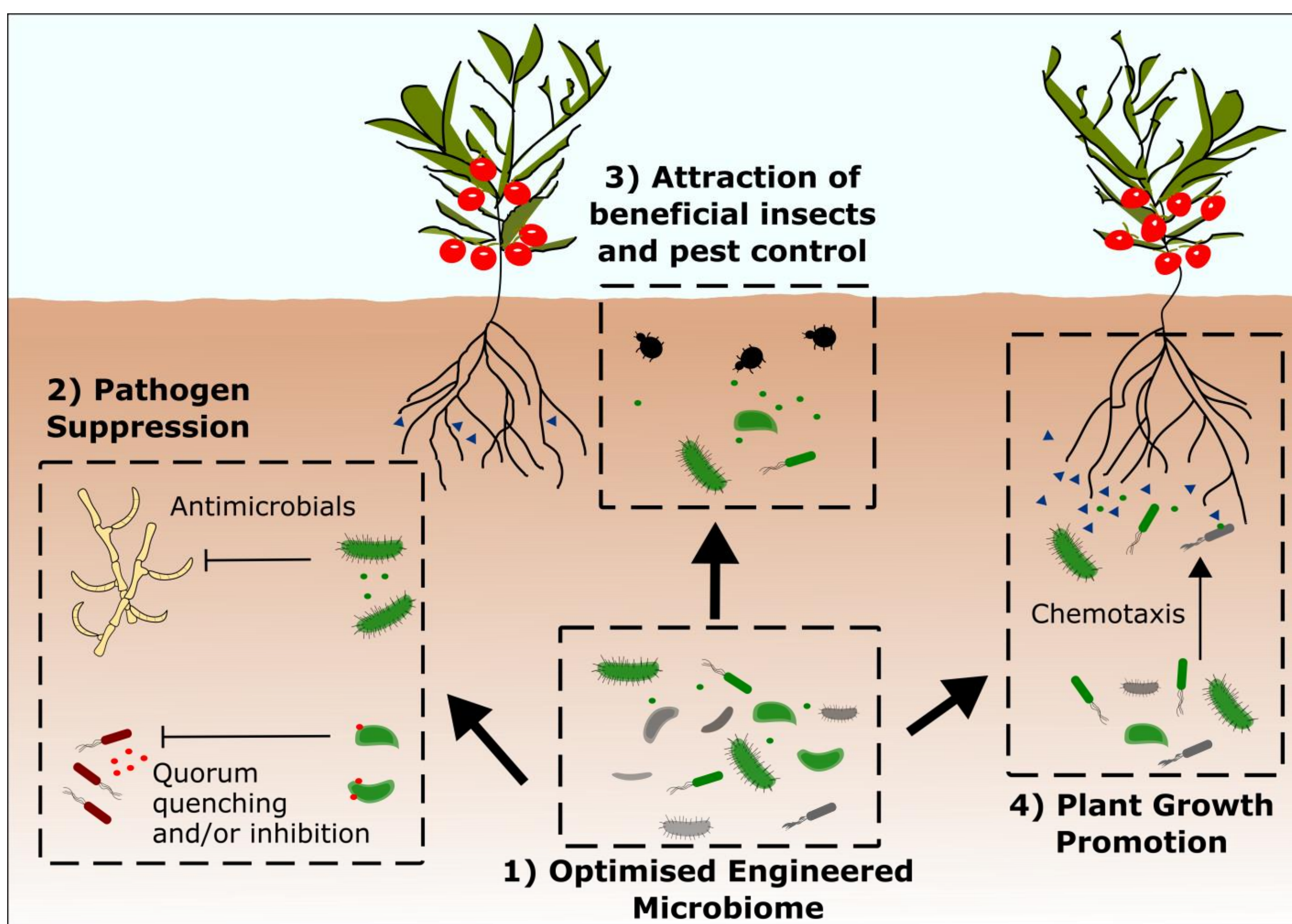

Figure 4 – Examples of potential benefits of a signal-optimised microbiome in agricultural applications[35]. 1) Intercellular signalling by genetically engineered microbes modulates and stabilises the population structure of a microbiome, including native and inoculated species, to regulate plant-beneficial outcomes. 2) Detection of plant pathogens in the soil by engineered microbes activates signal-specific disease suppression, with effective antimicrobials produced to inhibit growth. Intercellular lactone signalling regulating virulence factors is sensed by engineered surveillance bacteria and inhibited by quenching enzymes. 3) Engineered microbes produce volatile compounds that attract plant-beneficial insects, repel pests and dissuade feeding. 4) Engineered microbes undergo chemotaxis towards crop root exudates, aiding persistence of the engineered strains. Multiple mechanisms of plant growth promotion are activated upon root exudate detection, such as growth hormone secretion, phosphate solubilisation and nitrogen fixation.

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Molecular biologists are no longer limited to sourcing an organism to amplify a genetic part of interest by PCR. Part libraries are available, including, e.g., the BioBricks repository maintained by iGEM that includes many studied signalling systems[127]. In addition, the *de novo* synthesis of DNA by a variety of biotechnology companies is quickly becoming more accessible and affordable. This allows unprecedented access to sequence space, which, when combined with our bacterial genetic editing capabilities, allows real freedom in signal engineering.

4.3 Identification and development of parts for signal engineering

The possibilities for signal engineering are not limited to genes as they naturally occur, as elegantly demonstrated in *E. coli* in a study involving the inner membrane sensor PhoQ and the regulator it phosphorylates, PhoP[128]. Random mutagenesis was performed of amino acids in the interface of both proteins, and the resulting library screened at high throughput for response to Mg^{2+} levels using a *yfp* reporter gene assayed with flow cytometry. The former strategy allowed for the generation of 58 insulated pathways, without crosstalk with other PhoQ/PhoP pathway variants, effectively expanding the possibilities for differentially engineering the regulatory circuits of many genes at once. This can also be achieved through the use of natural systems that do not crosstalk, for example with the concurrent use of AHL and GBL signalling[129]. Natural enzymes can be altered through structure-informed rational engineering and directed evolution, as demonstrated for lactonases with altered substrate specificity[130] and increased quorum quenching activity[131,132]. These advances allow us to develop enzymes to perform functions for which naturally occurring enzymes are not available. This could allow us to develop multiple concurrent signalling pathways that interact in defined ways, whilst also expanding the possibilities for effector genes that respond to such pathways; for example, synthetic biology could provide the new biosynthesis route *in vivo* to important plant hormones.

4.4 Beneficial outcomes from the application of an optimised microbiome

There are a variety of studies that demonstrate the diverse outcomes achievable through engineering signalling. Social interactions within a bacterial community have been artificially generated, using the antimicrobial nisin as an intercellular signal[133]. These included enforced cooperation, where the two bacterial strains co-operatively biosynthesise nisin, which subsequently induces tetracycline resistance in both partners to allow survival under selection[134]. The possibilities for engineering soil microbiomes are not limited to inter-bacterial signalling: trans-kingdom signal genetic engineering has been achieved, with the expression of a heterologous biosynthetic pathway to the signalling molecule *scyllo*-inosamine in plants[135]. The signals produced by these transgenic plants were detected by rhizobial bacteria carrying the rhizopine lux biosensor. This represents an important foundational advance towards the use of synthetic biology to engineer plant–microbiome signalling pathways at the molecular level. Engineering to suppress a pathogen has been demonstrated in *E. coli*, which was successfully engineered to both produce an antibiotic and self-lyse in response to a *Pseudomonas aeruginosa* AHL[136]. Indeed, disease suppression could be a relatively straightforward application of signal engineering, whether through interruption of virulence factor QS or the induction of microbial antibiotic production. Alternatively, bacteria can be engineered directly for plant growth promotion, via mechanisms such as nitrogen fixation. In the corn root isolate *Kosakonia sacchari*, the regulatory network for the nitrogen fixation operon (*nif*) has been engineered to optimise nitrogen fixation in corn[137]. This has also been demonstrated with the introduction of the *nif* pathway for nitrogen fixation to two cereal endophytes as well as *Pseudomonas protegens* Pf-5[138], initially under IPTG inducible regulation. This demonstrates how synthetic biology allows us to introduce pathways that encode plant beneficial functions to heterologous bacteria. Furthermore, using a salicyclic acid sensor to drive the *nif* pathway yielded a 1000-fold induction of nitrogenase activity. Salicyclic acid and other root exudates could be used as signals to denote proximity to the crop, and selectively activate relevant genetic pathways in the bacterium. There is also the potential to regulate the relative populations of bacteria within the microbiome by artificial signalling; multiple QS systems introduced in tandem in *E. coli* have been used to regulate cell growth and populations in laboratory co-culture[139]. *E. coli* has also been engineered to sense and undergo chemotaxis towards hydrogen peroxide[140]. The same ideas could be applied to an engineered microbe in a crop soil microbiome, for instance to promote chemotaxis towards the crop root exudates, which could also help increase the persistence of the introduced bacteria.

5 Conclusions

To build an optimised microbiome, combinations of members would need to be selected and developed for persistence in field conditions and the robust exchange of signals to maintain the expression of functions critical to plant health and growth. This could be supported by genetic engineering of signal biosynthesis, degradation, and response circuits in some or all members of the engineered community, or within/by the plant host. Modern synthetic biology techniques provide the means to develop and install the parts needed for such systems. This engineered microbiome could inhibit pathogenic intercellular signals or sense them and specifically respond to provide antagonism. Bacteria could be engineered to undergo chemotaxis towards plant root exudates, followed by activation of plant growth promoting functions. The potential benefits of the application of such a signal-optimised microbiome are summarised in Figure 4.

This signal-optimised microbiome would be highly synergistic with plant host-mediated selection approaches, based on the evolution of enhanced microbiomes in response to artificial selective pressure towards a trait of interest[141–143]. Selection of the starting microbiome for such experiments is essential to their success[27,144,145], and a signal-optimised

microbiome would prove an excellent starting point. Microbes have the potential for mitigating the negative environmental impacts of agriculture, enhancing plant productivity, increasing plant resilience to environmental stress and reducing reliance on external fertiliser and pesticide inputs. Engineered microbiomes that capitalise on a deep understanding of the complex interactions within soil and plant microbiomes are needed to optimise the functional capacity of microbiomes to support crop and ecosystem productivity.

6 Author contributions

JAC, ET and RB conceptualised the article. ET, RB, WRH, MJS & LLK secured funding as part of the NSF/UKRI Signals in the Soil program. JAC, ET and RB created the original draft, all authors reviewed, contributed to and edited the manuscript.

7 Conflicts of interest

LLK is Chief Science Officer of Jord BioScience, and WRH and MJS serve on the Jord BioScience Scientific Advisory Board. Jord BioScience creates signal-optimised microbial consortia for use in agriculture. This interest has been reviewed and managed by the University of Minnesota in accordance with its conflict of interest policies.

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