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From axenic to mixed cultures: Technological advances accelerating a microbiology paradigm shift

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Abstract

Since the onset of microbiology in the late 19th century, scientists have been growing microorganisms almost exclusively as pure cultures, resulting in a limited and biased view of the microbial world. Only a paradigm shift in cultivation techniques – from axenic to mixed cultures – can allow a full comprehension of the (chemical) communication of microorganisms, with profound consequences for natural product discovery, microbial ecology, symbiosis, and pathogenesis, to name a few areas. Three main technical advances during the last decade are fueling the realization of this revolution in microbiology: microfluidics, next-generation 3D bioprinting, and single-cell metabolomics. These technological advances can be implemented for large scale, systematic co-cultivation studies involving three or more microorganisms. In this review, we present recent trends in microbiology tools and discuss how these can be employed to decode the chemical language that microorganisms use to communicate.

Keywords

Co-cultivation devices, co-culture, microfluidic devices, natural product discovery, microbial secondary metabolism, microbial communication

1. It is time for new discoveries

The biochemist and Sci-Fi author Isaac Asimov (1920-1992) said: *"The most common phrase to hear in science, those who heralds new discoveries, is not 'Eureka!' but 'What? This is funny.'"* It is tempting to think that this is what bacteriologist Alexander Fleming (1881-1955) said following his infamous, fortuitous observation of a *Penicillium chrysogenum* mold contamination on a staphylococcal plate [1], leading to the discovery of the β-lactam penicillin and saving millions of lives ever since. Arguably, the field of natural product discovery is more subject to serendipitous findings than other research areas [2], highlighting the need for holistic approaches in the quest of new **bioactive substances** (see Glossary). Currently, screening efforts have been drastically diminished, and the release of antimicrobials in the market has dropped alarmingly [3–5]. Also, candidate molecules and lead compounds often reveal themselves as well-known chemicals [2,6], showing the importance of **dereplication** efforts. These trends unfortunately coincide with the rise of bacteria being resistant toward antimicrobials, including so-called "last-resort drugs" [7]. Chemical approaches such as high-throughput screenings of chemical libraries [8], *de novo* chemical synthesis [9], or biotransformation [10] generate new chemical diversity, yet the main and most promising source of antimicrobials remains **microbial secondary metabolism** [5,6,11]. However, at least 20 years have passed with no new classes of antimicrobials being identified and the antibiotics pipeline is thus running dry [12]. It is a matter of urgency that we respond to the rising number of multi-resistant bacteria and fungi [13] in a timely fashion. Yet sequencing of hundreds of microbial genomes revealed that many species, in particular filamentous bacteria and fungi, devote a substantial part of their genes (up to 10-15 %) to secondary metabolism, potentially encoding the "penicillin 2.0" of the new century [14,15]. Surprisingly, most of these genes are silent, i.e. not expressed under laboratory cultivation conditions [16]. One obvious explanation for this significant challenge is that standard microbial cultivations introduced by Robert Koch use **axenic cultures** [17], concomitant with environmental conditions that microorganisms never face in nature: excess of macro- and micronutrients, high water activity, constant temperature, buffered pH, and isolation from the rest of the microbial world. Therefore, a substantial part of microorganisms' secondary metabolites, especially those allowing them to interactions, communication, alliances, or conflicts with other species, are not produced. Recent technological advances in co-cultivation devices provide a tremendous window of opportunity to activate the silent microbial secondary metabolism

and facilitate the discovery of new bioactive substances by implementing high-throughput **co-cultivation screenings** (Box 1, Figure 1).

In this review, we present and discuss the impact of new microbiology devices which enable smart and novel co-cultivation experiments to be performed. We argue that co-cultivation experiments can be implemented, and, if integrated with other emerging tools such as bioprinting and single-cell analytics, hold great promise to understand microbial interactions, specifically in the field of natural product discovery. Systematic investigations of multispecies microbial communities in a combinatorial way have, to the best of our knowledge, not yet been undertaken with miniaturized devices. Successful culturing of multispecies communities in this manner will likely have a huge impact on the discovery of new bioactive substances of microbial origin and will help to shed light into **biosynthetic dark matter**.

2. A glimpse in the dark: On microbial secondary metabolism and its role in nature

Microbial secondary metabolites are small molecules often secreted into the extracellular space and produced upon stress conditions and/or after entering the post-exponential growth phase. Although the secondary metabolism of microorganisms is not essential for growth and reproduction in axenic cultures, it is interconnected with the nutritional status, the general metabolic activity, and the developmental stage [18,19]. Logically, it must be coupled with primary metabolism, which, in contrast, is essential for growth and reproduction (Box 2). Primed with simple but ubiquitous cellular building blocks, such as amino acids or short-chain carboxylic acids, bacterial and fungal secondary metabolites form a bouquet of unusual and complex chemical structures harbouring bridged rings, heteropolycyclic or macrolide backbones, as well as cyclic peptides, which can be decorated with a diverse set of functional groups (Figure 2) [20]. Chemically, secondary metabolites are mainly classified into non-ribosomal peptides, polyketides, terpenes, alkaloids, and ribosomally synthesized and post-translationally modified peptides such as lanthipeptides [20–23]. Functionally, they are bioactive molecules, some of which have been shown to be pigments, chromophores, **siderophores** or melanins [24]. Their activities as antimicrobials, anti-tumorals, immunosuppressants, cholesterol-lowering agents or toxins makes them attractive for chemical, pharmaceutical, agricultural and food industries [13]. Given the very specific function of secondary metabolism, the term “specialized metabolism” might be more

appropriate, as has been suggested recently [25]. Similarly, the general term “antibiotic” (from the Greek “anti,” against, and “biosis,” life) to describe antibacterial and sometimes antifungal drugs is not doing justice to the tremendous range of activities and targets that these bioactive molecules possess. Recent studies showed that at sub-inhibitory concentrations antibiotics activate expression of a large subset of genes, including those involved in biofilm formation and virulence [26–28]. Other works have most recently demonstrated that antibiotics act as inducing agents to enable discovery of new ones when added exogenously to microbial cultures [29]. This raises the exciting hypothesis that antibiotics in nature act not only as killing agents but more likely also as signaling molecules and/or **hermetic substances** crucial to shape the interaction and relationship among microbes. Thinking of antibiotics in this way challenges our anthropocentric view of nature. “*Dosis sola facit venenum*” (the dose makes the poison) once said Paracelsus. He might also be right in this context.

3. A shot in the dark: Approaches to activate the silent microbial natural product reservoir

Actinobacteria and filamentous fungi possess up to 40-80 **biosynthetic gene clusters (BGCs)** in each genome, which are predicted to encode the necessary enzymes required for the synthesis of secondary metabolites; however, only a small fraction of these compounds have been chemically characterized and linked to specific BGCs. At present, some hundreds of secondary metabolites of bacterial and fungal origin have been described [15,21,30–34]. Currently, most approaches to activate the silent microbial secondary metabolism are performed in axenic cultures and are either targeted (e.g. activation of a pathway-specific transcriptional factor) or non-targeted (e.g. activation of epigenetic factors) [35,36]. Whereas the former require *a priori* knowledge of a specific BGC [37–39], the latter are less-specific and modify global gene expression with direct and indirect consequences on the expression of multiple BGCs [40–47]. Further pleiotropic approaches rely on variations of the growth media (e.g. **OSMAC approach** [48]). Only a few try to mimic (inter-kingdom) microbial interactions in nature by means of co-cultivations on defined media [49–53].

As opposed to primary metabolism, the correlation between secondary metabolism genes and their products is not straightforward; not only BGCs are often silent, but it is cumbersome to associate secondary metabolism profiles with BGCs [30,54]. Bioinformatic tools (e.g. SMURF, AntiSMASH) for the identification of BGC and/or their

products [6,55–57] as well as other approaches like the “genomisotopic” ones [58], are useful in generating a *chemotype-to-genotype* or *genotype-to-chemotype* correlation [6,59]. However, many BGCs, besides the **core or key enzymes** for the synthesis of the secondary metabolite “backbone”, possess tailoring enzymes that decorate (e.g. glycosylate or prenylate) the secondary metabolite with chemical modifications generating further chemotypes [60], which would be hard to predict by bioinformatics. A further layer of complexity is added by the fact that two distinct key enzymes might synthesize the same secondary metabolite (as in the case of the two NRPS-like proteins encoded by *atmelA* and *apvA* in *A. terreus*, which both produce aspulvinone E that, depending on the cell type, is converted into melanin or aspulvinones if localised into the conidia or hyphae, respectively [61]). Moreover, some key enzymes can participate in the **“natural combinatorial biosynthesis”** of several secondary metabolites (as in the synthesis of three pyrrolamide antibiotics by two BGCs in *Streptomyces netropsis* DSM40864, i.e. congocidine, distamycin and a congocidine/distamycin hybrid [62]). An extreme example of this cross chemistry has been documented for a tripartite, inter-kingdom bacterium-fungus-plant association. Synthesis of the polyketide-derived phytotoxin rhizoxin by the rice seedling blight fungus *Rhizopus microsporus* was shown to be dependent from its own endosymbiontic bacterium *Burkholderia* sp. [63]. Recently, rhizoxin was shown to be modified by an enzyme of the fungal pathogen by adding an oxirane (epoxide) ring and, most importantly, that this modification is not involved with drug detoxification but with toxicity enhancements toward the host plant *Oryza sativa* [64]. The extent of this natural combinatorial synthesis among different species, which is less studied than approaches involving heterologous cloning of biosynthetic genes (see e.g. [65]), cannot be predicted; even with a conservative estimate, many millions bioactive microbial secondary metabolites potentially exist [11,13,36].

A parallelism could be drawn with the surpassing of the “one-gene-one-enzyme” hypothesis in the advancement of our understanding of molecular biology and the regulation of gene expression. Overcoming the paradigm “one-BGC-one-secondary metabolite” might prove crucial for the discovery of new secondary metabolites. Co-cultivation studies, in particular when conducted with the appropriate microbiological tools, will arguably prove crucial to investigate the silent microbial natural product reservoir.

4. Understanding life at the microscopic scale

In vitro cultivation of microorganisms as axenic cultures, and the fact that the overwhelming majority of microorganisms does not grow in the laboratory, results in both a limited and biased view of the microbial world [25]. Growth of microorganisms at the microscopic scale, where in particular viscosity, diffusion and surface tension play a crucial role, is dictated by different physical laws when compared to shaken flasks or Petri dishes [66]. It was elegantly shown 40 years ago that at the micrometre scale, high viscosity causes bacteria to move more slowly than diffusing nutrients, resulting in a passive foraging food strategy [67]. The flux of molecules and nutrients in nature is certainly not as homogeneous and reproducible as under laboratory growth conditions [68]. This should result in even higher cellular heterogenicity in populations of microorganisms, a phenomenon which is recurrently observed and studied in the laboratory (e.g. [69]). This exerts a further layer of complexity that should be considered when studying the coordination of microbial gene expression with abiotic and biotic environmental stimuli. It is important to note how studies that have been successful in isolating and growing the **"microbial dark matter"** are often followed by the sequential passaging (also referred as "subculturing") of cells as pure cultures, thus resulting in **domestication** of them (e.g. [70–72]). All this undermines our knowledge and understanding on the central role of microbial interactions in nature.

Due to the difficult execution with existing tools, *in vitro* cultivation of microorganisms as mixed cultures – be it for antibiotic discovery [53], in the design of synthetic consortia for metabolic engineering purposes [73–75], or in the study of interactions among environmental isolates [76] – has been so far mostly limited to bi- or tripartite association studies [77]. Main challenges for the co-cultivation of microorganisms involve the uneven growth rate of the strains, as well as the different nutrient requirements or abiotic incubation conditions. Successful studies involve mixing different media, application of growth parameters that are suitable for both co-cultivated partners, and/or the pre-growth of one of the two species to account for different growth rates. However, it is obvious that to dissect all the possible chemical and physical interactions when dealing with multi-species communities, essentially limitless combinatorial possibilities for parameter optimization arise. Consequently, co-cultivation often represents both a prerequisite, and a bottleneck, to understand microbial ecology, symbiosis, secondary metabolism, and/or pathogenicity [50,76,78–82].

5. Current microbiological tools and their potential use in co-cultivation studies

Given the current (r)evolution of microbiological techniques and the recent exponential-like increase in microbial co-cultivation studies (Figure 1), tools for co-cultivation of microorganisms are still in their infancy. Novel, promising microbiological advances, and their possible uses in co-cultivation studies, are summarized in Table 1 and discussed below.

Classical cultivations and miniaturized versions thereof

Classical techniques remain the “gold standard” for microbiology experiments to grow, maintain, or domesticate strains. They are easy to perform and allow the isolation of sufficient amounts of secondary metabolites for subsequent chemical analysis, but also require considerable amount of consumables (media, materials) and time. Co-cultivations studies are often performed based on serendipitous associations of strains/species [81], educated guesses [51], or are painstakingly executed by bi-partite screenings [50]. A bias toward cultivable, well-studied species known to produce many secondary metabolites (e.g. *Streptomyces* spp. or *Aspergillus* spp.) is observed.

Classical methods are successfully used for the screening of new isolates with antimicrobial activity. Kawaguchi *et al*. [83] combined the plating of soil-derived fungi with a bioactivity screening against *Candida albicans*. A separation of the strains/species after co-cultivation is often difficult, but can be achieved with further tools (e.g. semi-permeable membranes [72,84] or dialysis culture flasks for the physical separation of cells while maintaining chemical contact [85]).Miniaturization of classical techniques facilitates the execution of co-cultivation experiments. The Biolog System [86], for example, which is used to characterize the phenotype of strains growing in different chemical environment in 96-well plate format, can be used to assess the influence of varying abiotic conditions on co-cultivated species or characterize the physiology of different complex microbial communities (e.g. [87]). With the development of micro-Petri dishes, Ingham *et al.* [88] created a porous ceramic chip (36x8 cm) that can be placed on top of a regular agar plate and be used for high-throughput screenings. Embedding or streaking a co-cultivation partner in/on the bottom agar would allow the high throughput, pairwise screening of the chemical interaction with the cells in the micro-compartments. The integration of classical plating techniques with nanospray desorption electrospray ionization (NanoDESI) and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF), is used in the so-called imaging mass

spectrometry (IMS) to investigate chemical signatures of interspecies microbial interactions [89,90].

Microfluidic devices

Microfluidic devices [66,91] are tools that allow the handling of liquids in µm to pm scale to create liquid-liquid interfaces (with miscible and immiscible fluids), e.g. using laminar flows. The devices are often designed with computer-aided design (CAD) software and produced by engraving, micromachining or moulding of materials as silicones, ceramics or acrylic glasses. They are often connected with microscopy and permit single-cell analytics as well as parallel, miniaturized experiments, holding great potential for co-cultivation studies. Physical conditions in microfluidic devices are more controllable and representative for life at the microscopic scale, while miniaturization allows parallel experiments and high surface area-to-volume ratio, which facilitates diffusion of secreted metabolites in microsystems. Automation might be foreseen, and the devices are also sometimes referred as “Lab-on-a-chip” or microelectromechanical systems (MEMS) [92]. The small working volumes and the capability of microfluidic devices to precisely control growth dynamics, e.g. through the flow of media and device-specific physical micro-constrictions, make them suitable for single-cell analysis as well as investigation of microbial community assembly [93–96].

Hesselman *et al.* [97] developed a reusable, two-compartment device for co-cultivation experiments between *Escherichia coli,* and the nematode *Caenorhabditis elegans,* which were separated by microsieves. An open microfluidic platform (a.k.a. suspended microfluidic) based on liquid surface tension, and capillary flow, was shown by Casavant *et al.* [98] to be suitable to investigate chemotaxis in eukaryotic cells. The air-liquid interface facilitates the extraction of metabolites, while multiplexing of capillaries (“µDot device”) generates several distinct compartments within the platform. By using hydrogels between the capillaries, chemical and physical contacts can be maintained and prevented, respectively. By designing a high-throughput microfluidic platform with hundreds of physically separated, flow-through chambers, connected with time-lapse microscopy, Grünberger *et al.* [93] generated single-cell data with spatiotemporal resolution, including morphology and cell division dynamics, for *Corynebacterium glutamicum*. Importantly, the authors later showed the suitability of a similar device to investigate population heterogeneity in the filamentous fungus *P. chrysogenum* [94]. Due to flow-through of media in the microfabricated device, one condition at the time

can be investigated, which is generally comparable to classical co-cultivation studies. Uniquely however, miniaturization allows feeding with pulses of different media, or facilitates the analysis of downstream, inter-species effects of secreted chemicals (e.g. with the sequential combinatorial arrangements of species along the flow-through direction). In microfluidic devices, especially those with several hundred compartments, cells are “brought” into place by dip-loading and capillary action, microinjection, or seeding of cells into micro-compartments, hence often relying on the stochastic inoculation of cells. The major challenge for co-cultivation screenings using microfluidic devices would be to precisely inoculate different combinations of complex microbial consortia into miniaturized devices with hundreds of distinct compartments.

Encapsulation techniques

A special version of microfluidics is encapsulation technology, where droplets are dispersed in different phases (e.g. water-oil-water). Droplet-based approaches rely on small volumes of fluids and therefore could be considered as a subset of microfluidic approaches. Micro-compartments are generated by confining cells in emulsions of agarose-based, aqueous or gel (e.g. polymeric compounds like PDMS) microdroplets. Microdroplets allow diffusion of molecules and are sessile, semi-permeable containers (with both size and hydrophobicity of chemicals influencing diffusion). Multiple species, as well as single cells, can be encapsulated in microdroplets, thus allowing investigation of promiscuous physicochemical (cell-cell) contacts or of indirect chemical interactions without physical ones. Being physicochemically confined, cells cannot escape the encapsulation, while the size of droplets can be controlled by osmotic diffusion of water [99].

First described in the 1950s in a seminal paper to observe growth and motility of single cells [100], the technology was further developed to grow **uncultured microorganisms** under low-nutrient media in percolating columns [101], or to perform high-throughput chemical sensitivity screenings [102]. The encapsulation approach has proven useful to investigate synergistic effects of microorganisms in bi- and tri-partite assays [103], where the authors showed that spatial organization of microorganisms is involved in the establishment of **syntrophy**. Recently, Niepa *et al.* [99] probed bacterial-fungal interactions and demonstrated antagonistic dynamics between *P. aeruginosa* and *C. albicans*, showing differences upon physicochemical (eradication *C. albicans* upon co-localization of *P. aeruginosa*) and chemical interactions of the species (repression of

filamentous growth of the fungus when the bacterium is excluded from the microdroplets).

The SplitChip [104], in which cells grow in compartments that are subsequently split into two, relies on microfluidics and could be considered as a miniaturized version of replica plating. Originally developed for differential analysis of the split compartments, i.e. for both scale-up (e.g. to grow new uncultured species) and destructive analysis (e.g. molecular methods like colony PCR), the technology could be useful in co-cultivation experiments. For example, upon splitting, metabolomics analysis of one split compartment, or transplantation of microbial communities, can be easily done.

Using the microfluidic streak plate [105], high-throughput cultivation of cells in nL-volumes in regular petri dishes filled with an inert carrier oil by manual or robotic streaking can be achieved. This technology was used to identify a complex microbial community within a droplet able to degrade polycyclic aromatic hydrocarbons. As for microfluidic approaches, the encapsulation of cells – especially when dealing with complex environmental samples – might be dictated by chance. Even if this issue is overcome by the large amount of droplets that can be generated and screened, a way to modulate droplets dynamics would represent a great advantage for co-cultivation screenings. While volume, composition and stability of microdroplets can be manipulated [106], their precise orientation/localization in space cannot yet be controlled.

3D-bioprinting

Modulating the position of microdroplets might be superfluous when microbial communities become established via 3D-bioprinting, where complex structures can be designed in any desired geometry using a gelatinous matrix [107]. Investigating nonsperoidal geometries of bi-partite microbial communities, a recent study demonstrated that nesting *Staphylococcus aureus* within structured shells of *P. aeruginosa* increases resistance of the first toward *β*-lactams [107]. The porous nature of the matrix and the versatility in producing any desired geometry makes the technique attractive to study structured microbial communities.

6. Concluding Remarks

"Prediction is very difficult, especially about the future" said the physicist Niels Bohr (1885-1962). If one is to extrapolate from the present trend of increasing studies on

microbial co-culture and the currently available toolset (Table 1), and to judge the potential hidden in the "microbial/biosynthetic dark matter," then the field of microbial co-cultivation holds great promises for the future and will continue to thrive. The road to natural product discovery is long and arduous, and microbial cultivation is just *one* bottleneck in drug discovery, which includes elucidation of bioactivity mechanism and chemical structure as well as clinical trials. We argue that since microbial (co-)cultivations are often the initial step in drug discovery, implementation of co-cultivation tools will benefit the whole pipeline.

Borrowing a concept from computer science, the **Moore's Law of Microbiology** has been formulated [108], drawing a parallelism between the miniaturization of microbial cultures and the number of transistors per chip in microelectronics. Microfabrication holds great promise for microbiology [109], and the single cell size limit will be reached earlier than the single atom limit in microelectronics [108]. However, device compartments need to accommodate complex, multi-microbial communities resembling natural ones, and miniaturization *per se* is not the only pivotal factor for the execution of co-cultivation experiments (Figure 3). Integration with downstream analysis is crucial, as well as the ability to discriminate between chemical and physicochemical effect among promiscuous cultures. For example, the open microscale platform by Barkal *et al.* [110] is a microtiter plate-size device investigating the effect of culture microenvironments during microbial (co-)cultivations, with an integrated metabolite extraction platform facilitating downstream analytics. Importantly, the authors showed that different geometries of the compartments influence the profile of secondary metabolites produced by *A. nidulans*, and implemented the device to allow co-cultivation of e.g. the plant pathogen *Ralstonia solonacerum* and *A. flavus* [110].

Engineering of microbial consortia is, although technical challenging, implementable for industrial and biotech purposes [111,112]. The group of Akio Ozaki showed large-scale production of commercially-valuable mono- and oligosaccharides by tri-partite cultures of recombinant *E. coli* strains and *Corynebacterium ammoniagenes* [113,114]. Ying-Jin Yuan and colleagues used co-cultures of *Ketogulonigenium vulgare* and *Bacillus megaterium* for the industrial production of vitamin C [115], thus validating the use of stable, large-scale multispecies consortia of microorganisms for applied purposes.

An aspect that should be considered is that natural products are often uncovered by studying the associations of microorganisms with plants [116] or insects [117,118].

Some of the tools discussed in this review have been shown to be useful to grow nematodes [97], in dissecting the effect of fungal secondary metabolites on zebrafish (vertebrate) embryos [119], or study chemoattraction and 3D-growth of cancer cells [98], which opens new exciting prospects for the study of microbial interactions with multicellular eukaryotes. It is argued how a theoretical framework [120] and the integration of experimental data with mathematical modelling [121] would largely benefit the fields of microbial ecology and mixed-culture studies. We believe that co-cultivation experiments mostly neglect synergistic interactions among microorganisms as well as the role of volatile compounds as signaling molecules (see Outstanding Questions) [122,123].

Crucially, co-cultivation experiments can be done, as opposed to molecular approaches, without extensive knowledge of the strains used [2,6]. Serendipity in natural product discovery, and by extension in science, is not to equate to sheer luck. Creating the nourishing environment for breakthrough discovery by having the appropriate tools, theoretical framework or design of experiment is very much a prerequisite. We are convinced that implementation of current microbiology tools and their application in co-cultivation screenings will be a turning point for natural product discovery. In line with Isaac Asimov, we dare to predict that scientists on their way to new discoveries in the secondary metabolism of microbes will increasingly say *“What?”* again.

Trends

A limited, biased, and anthropocentric view of the microbial world with focus on fast-growing **copiotrophic** species has emerged from classical axenic cultivation approaches.

Recent (meta)genomic insights unveiled the potential hidden in microbial diversity. However, cultivation-independent approaches cannot replace cultivation techniques. Cultivation techniques have to evolve further – from axenic to mixed cultures – to fully understand the microbial world.

Newly emerged tools including microfluidics, bioprinting, high-throughput screening, and single-cell analytics need to be fully implemented and integrated with existing (microbiology) techniques to systematically investigate and exploit microbial co-cultures.

Outstanding Questions

Which opportunities and challenges offer miniaturization of microbiology tools for co-cultivation studies? How can reproducibility of results, as well as stability of complex microbial communities, be guaranteed as is the case for classical, macroscopic experiments? Can miniaturization address these issues by the execution of multiple, parallel experiments? Is reaching the single cell limit in microcompartments hindering the investigation of complex, multispecies consortia?

Which tools to systematically investigate multi-partite microbial associations in co-cultivation screenings (i.e. with more than two or three strains/species) will be established? How can uncultured species be grown/exploited without domestication steps, thus unleashing the potential hidden in the biosynthetic dark matter? Would new tools allow the investigation of non-antibiotic effects of secondary metabolites at sub-inhibitory concentrations?

How will techniques like metabolomics and other “-omics” techniques, microscopy, IMS be increasingly integrated with the proper theoretical framework for the systematic investigation of complex microbial interactions? How can miniaturized experiments be up-scaled to validate the results and if necessary to produce sufficient amounts of induced secondary metabolites?

How can an effective design of experiment (DoE) to activate the silent secondary metabolism of microorganisms by co-cultivation experiments be ideated, taking into consideration the effects of physicochemical (cell-cell) and chemical signals (diffusible secondary metabolites, including volatile compounds) as well as microscale geometries and spatial structures of microbial communities?

Glossary

Axenic culture: pure culture of microorganisms, i.e. of only one species/strain.

Bioactive substances: chemical molecules showing bioactivity e.g. as antimicrobials, anti-tumour agents, immunosuppressants or anti-cholesterol agents. Antimicrobials (often referred to as antibiotics, antifungals and/or antibacterials) specifically kill or inhibit growth of fungi or bacteria.

Biosynthetic dark matter: are the unknown products of silent BGC of known species and, by extension, putative new bioactive substances from the uncultured microbial diversity.

Biosynthetic gene cluster (BGC): at least two physically-clustered genes encoding enzymes acting in concert in a biosynthesis pathway. Inactive BGCs are often referred as “silent,” “cryptic” or “orphan” genes.

Co-cultivation screenings: here we arbitrarily refer to this term to indicate the systematic, miniaturized and/or parallel investigation of co-culture of microorganisms where two, three or more species/strains can coexist, as opposed to “classical” studies often based on educated guesses and mainly investigating one bi-partite interaction at the time (e.g. in Erlenmeyer flasks or Petri dish).

Copiotroph: (micro-)organism that thrive in niches rich in available nutrients as opposed to oligotroph. Copiotrophic environments with nutrient-rich solutions are the standard cultivation media in microbiology whereas they are not prevalent in nature.

Core/key enzyme: the enzyme for the synthesis the secondary metabolite “backbone” which can be further modified by tailoring enzymes; usually, there is one key enzyme pro BGC, in particular the multi-domain enzymes non-ribosomal peptide synthetase (NRPS), polyketide synthase (PKS) or NRPS-PKS hybrid.

Dereplication: efforts to discover truly novel substances as opposed to the detection/isolation of known bioactive molecules, which is a recurrent phenomenon in natural product discovery.

Domestication: the step(s) employed to grow uncultured species. This often involves the sequential passaging of pure microcolonies on common laboratory media to obtain macroscopic colonies e.g. on a Petri dish.

Hormetic substance: chemical showing a dose-dependent effect on a target cell/organism as recently shown for antibiotics (i.e. enhancement of biofilm formation at

sub-inhibitory concentration while lethal effects at high dosage); the phenomenon is called hormesis.

Microbial dark matter: the fraction of microorganisms that cannot (yet) be cultivated in the laboratory.

Moore's Law of Microbiology: a parallelism between microelectronics and microbiology, comparing the predicted doubling, every two years, in the number of transistors per chip (microelectronics) with that of compartments per cultivation tool (microbiology). Also in microbiology, this trend is fueled by miniaturization/microfabrication.

Natural combinatorial synthesis: the synthesis of secondary metabolites by the cross chemistry of different BGCs within an organism or, possibly, among different species.

OSMAC: one-strain-many-compounds, an approach to increase the portfolio of secondary metabolites produced by one strain by varying the cultivation conditions.

Secondary metabolism: the branch of the cellular biochemical reactions that, as opposed to the primary metabolism, is not essential for growth, development, reproduction and basic cellular homeostasis. The products are called secondary metabolites (occasionally also referred to as idiolites, exometabolites or extrolites). Genes for the production of secondary metabolites are often organized in BGCs.

Siderophore: iron-chelating molecule increasing the solubility and thus bioavailability of extracellular, oxidized ferric iron.

Syntrophy: cross-feeding of two or more species/strains which show nutritional/metabolic interdependence.

Uncultured microorganism: also non-cultured, uncultivable, unculturable; microorganisms that fall into the "microbial dark matter." This is not synonymous with viable but nonculturable cells (VBNC), which are cells that due to metabolic imbalances or other unknown reasons enter into a physiologically inactive (dormant) state and are recalcitrant to growth on otherwise favorable media. Since both phenomena are not yet fully understood, a distinction is not always possible.

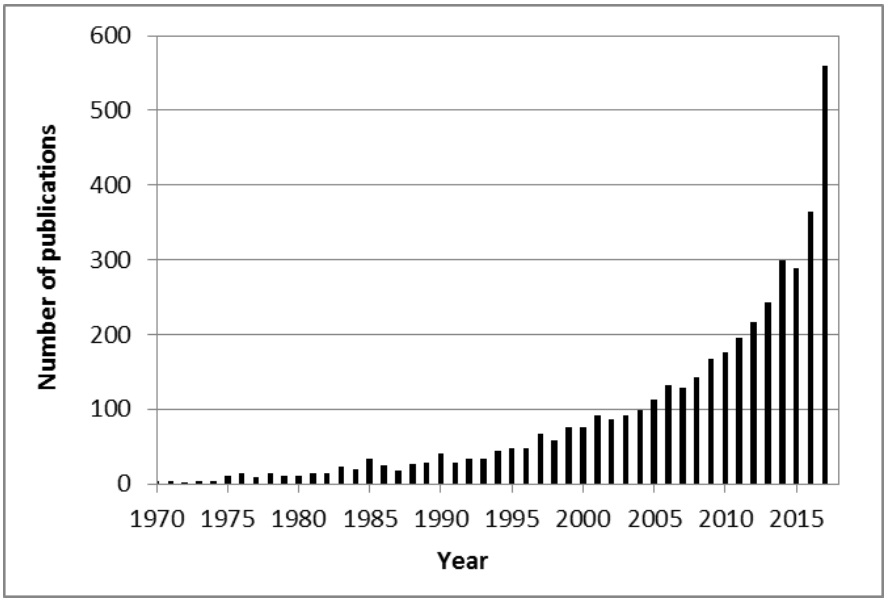

Figure 1. Exponential-like increase of publications on microbial co-cultivations in the last ~ 45 years. Plotted are the search results on PubMed with the query ("microorganisms"[TIAB] OR "microbial"[TIAB] OR "fungi"[TIAB] OR "fungal"[TIAB] OR "bacteria"[TIAB] OR "bacterial"[TIAB]) AND ("co-culture"[TIAB] OR "coculture"[TIAB] OR "mixed fermentation"[TIAB] OR "mixed culture"[TIAB] OR "combined culture"[TIAB] OR “co-cultivation”[TIAB]); only titles and abstracts were queried in the literature survey, resulting in 3’700 hits (as of March 2017). For 2017, an estimated number is given which we extrapolated from the ca. 140 papers published in the first 3 months. Please note that some studies, including articles discussed in the text, use a different terminology, in particular in the field of environmental microbiology, microbiome research, metabolic engineering or synthetic biology (e.g. “*in vitro* community reconstruction,” “species-specific/multispecies/interspecies/biotic interactions,” “one-to-one competition,” “microbial consortia engineering,” “polycultures,” etc.). Therefore, the number of publications is likely higher, especially for the last decade. Before 1970, only single-digit hits/year were obtained (20 in total; not shown).

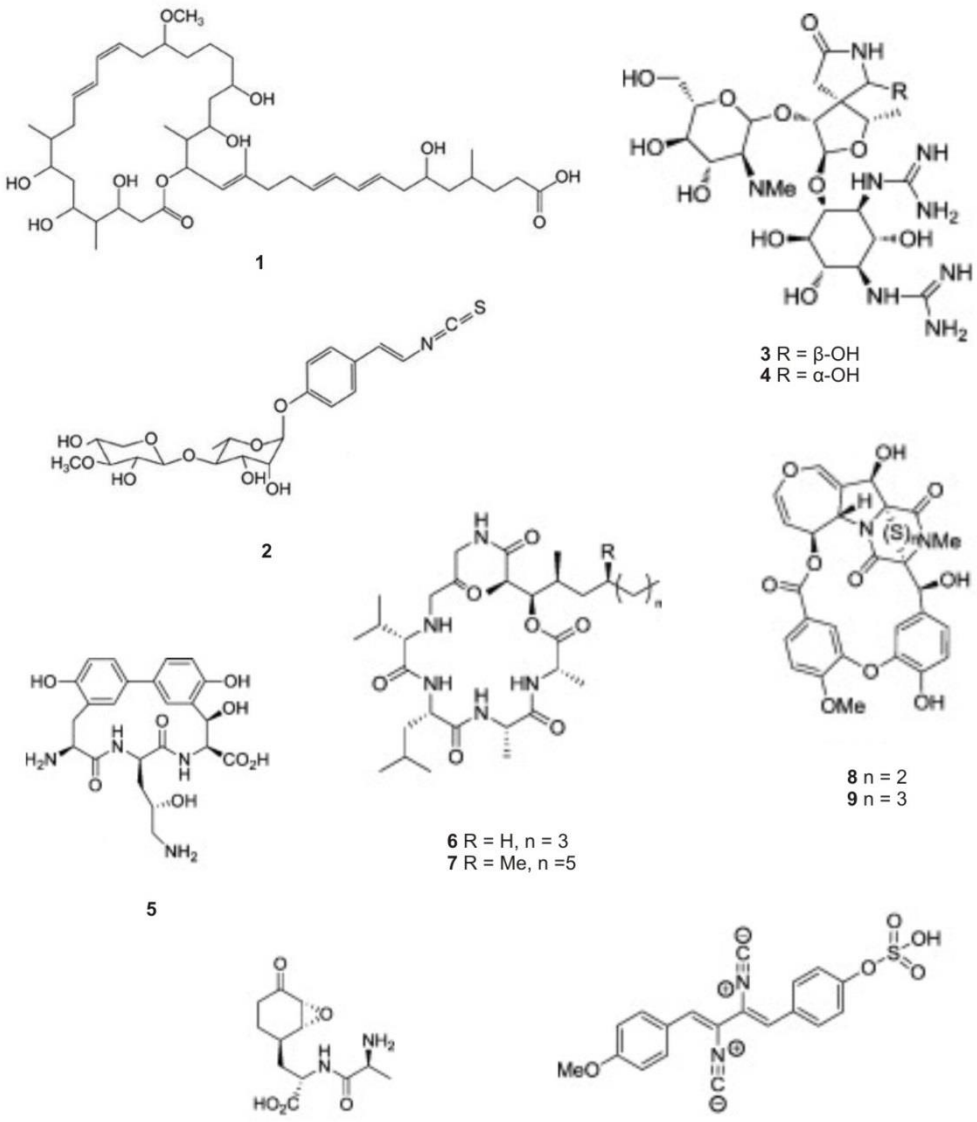

Figure 2. Selected examples of microbial secondary metabolites demonstrating the diversity of chemical structures. Shown are the natural products of fungal and bacterial origin **1** lagriene (polyketide), **2** sinapigladioside (aromatic glycoside with isothiocyanate group), **3**-**4** rhodostreptomycins A and B (aminoglycosides, isomers), **5** biphenomycin A (cyclic peptide), **6**-**7** emericellamides A and B (cyclodepsipeptides), **8**-**9** emestrins A and B (macrocyclic piperazine derivates), **10** bacilysin (non-ribosomal peptide with epoxide group) and **11** BU-4704 (xanthocillin analogue with cyanide groups). The secondary metabolites are induced by co-cultivation (for details see references [36,82]).

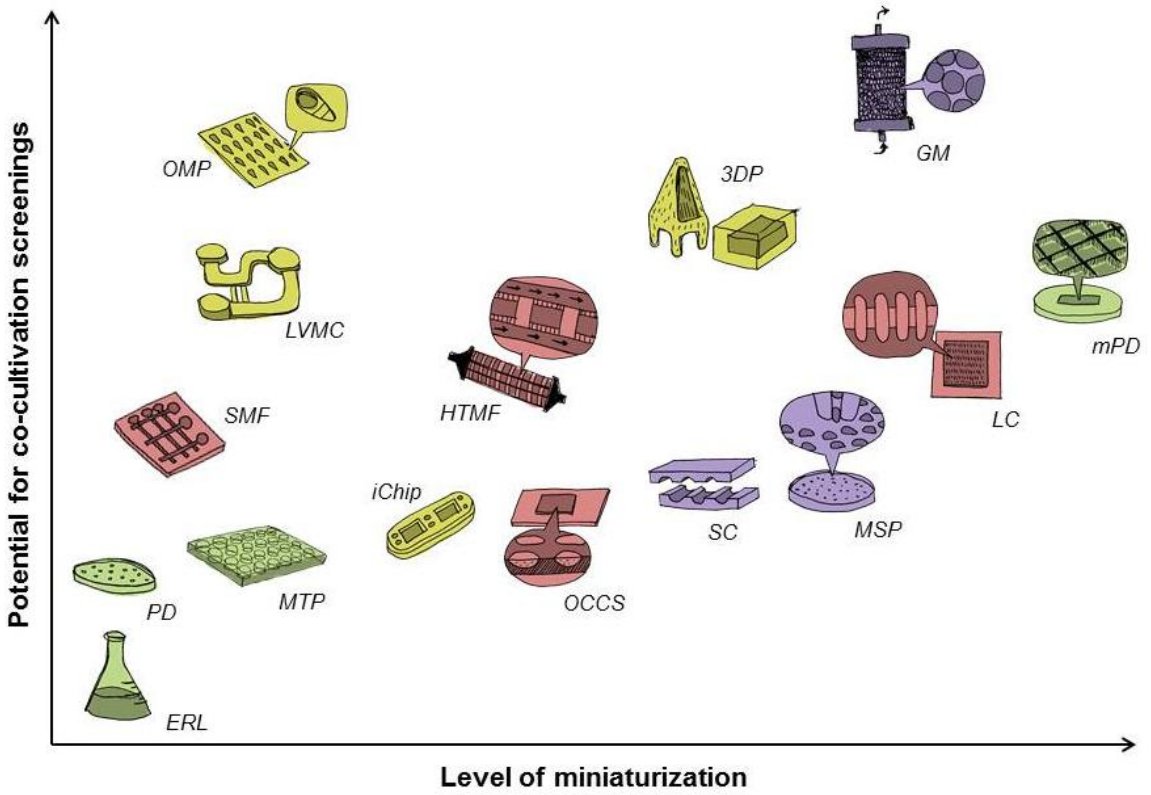

Figure 3. Selected microbiology tools and their suitability for co-cultivation studies. Shown are classical microbiology tools (green), microfluidic devices (red), encapsulation approaches (purple) and other tools (yellow). In addition to miniaturization, integrated techniques such as microscopy or metabolomics extraction increase potential for co-cultivation screenings. For details see text (please note that the devices are not in scale; level of miniaturization refers to the number of compartments and not to the size of the device or compartments). Abbreviations: PD: Petri dish; ERL: Erlenmeyer flask; MTP: microtiter plate; mPD: micro-Petri dish [88]; SMF: suspended microfluidics [98]; HTMF: high-throughput microfluidic [93]; OCCS: on chip culture system [124]; LC: Living Chip [92]; SC: Split Chip [104]; MSP: microfluidic streak plate [105]; GM: gel microdroplets [101,103]; iChip: isolation chip [84]; OMP: open microscale platform [110]; LVMC: low-volume migration chamber [119]; 3DP: 3D printing [107].

Key Table – Table 1. Overview of current microbiological tools and their amenability for co-cultivation studies.

Device or technique	Use in co-cultivation studies			Relevant refs.
	Pros	**Cons**	**Potential for implementation**	
Classical cultivations and miniaturized versions thereof				
Surface cultivation (Petri dishes) First introduced by Robert Koch and Julius Petri and still an irreplaceable step for microbiological analyses	Easy to perform; different plating geometries and compartmentalization possible; can be coupled with analytic techniques like IMS	A "common ground" (medium) for growth of both organisms required; experiments mostly based on educated guesses.	Very limited. Screenings often done to grow new species with a "helper" strain or identify antimicrobial activity	[72,83,89,125]
Submersive cultivation (Erlenmeyer flasks) Cells in liquid cultures under vigorous shaking to allow high mass transfer of nutrients and oxygen	Easy to perform; analysis of culture supernatant allows investigation of secreted chemicals; controls (e.g. with heat-deactivated cells) usually necessary	Same as above. Separation of cells/species after contacts difficult; different growth rates usually not taken into consideration.	Very limited. Dialysis culture flasks (described ca. 1900) with a semi-permeable membrane prevent cell-cell contacts	[85]
Microtiter plates 12- to 1536-well plates for miniaturized growth of cells (mostly static or with mild shaking in liquid, solid or viscous media)	Same as above	Same as above	Limited. Compartmentalization can be achieved with inserts (*transwells*); varying abiotic conditions can be assessed with the Biolog System	[86]
Micro-Petri dish A porous ceramic sheet (1 mio. compartments 7 x 7 µm) placed on top of agar; used for high-throughput screenings (enzyme-based, fluorescent) or high-density culturing	Presence of air-liquid interface favors oxygen transfer; placing on top of agar allow a reservoir for media, waste product and liquid (less evaporation) as well as transfers to different media	Stochastic inoculation by overflowing with cell suspension; Not very suitable for investigating tri-partite interactions	High, given that one can precisely inoculate cells into the micro-compartments; suitable for high-throughput, pair-wise screening with a co-cultivation partner in the bottom agar	[88]
Microfluidic devices				
Microfluidic cultivation platforms Microfluidic devices with flow-through of medium for single cell analysis of growth/morphology, usually based on trapping of single cells	Flow-through of liquid suitable for collecting and analyzing secreted metabolites	Microscopy is less informative on cell-cell interactions; controlled inoculation of multiple species/strains might prove challenging	Possible through serial combinations of single cell compartments (connection of flow-through) or (stochastic) trapping of multiple species	[93–96]
Multi-platform flow device Porous aluminum oxide microsieve (ca. 0,2 µm) connecting two flow-through channels	No need for membranes; chemical contact is guaranteed; fluorescence microscopy possible	Current design relies on educated guesses and by-partite co-cultivations; used to grow nematodes as well	Limited with the existing design; new designs are possible	[97]
On-chip culture system	Might allow fine-tuned analysis	Sealed chambers do not allow	Limited. Currently based on	[124]

Miniaturized chambers sealed with semi-permeable membrane for the isolated growth and microscopical analysis of single cells	of different ratio of co-cultivated species; media exchange through overflow/flow-through possible	exchange of metabolites among compartments of plasticity in experimental design (e.g. no reiterated opening and closing the system possible)	educated guesses of interacting partners or random inoculation of environmental samples; microscopy less informative on the specific interactions	
Microscale capillary flow A suspended microfluidic tool as open platform with an air-liquid interface	Multiplexing possible ("µDot" device); compartments can be physically separated by hydrogels (chemical contact maintained); multilayer biphasic system allows metabolomic analysis	Flow of fluids limited to the size of the chambers (i.e. no flow-through as in refs. [94,124])	High due to multiplexing of chambers, but not demonstrated yet	[98,126]
Capillaries Living Chip or GigaMatrix with 10,000-100,000 through-holes retaining fluid (ca. 50-200 nL) by capillary action; inoculation by dip-loading or microinjection	Through-holes can be inoculated differently by precise micro-injection; readily interfaced with microtiter plates (injection or downstream handling)	Read out by microscopy less informative for metabolic changes; stacking does not prevent cell-cell contacts	Might be high due to stacking of chips, yet still speculative; growth of filamentous species might be problematic	[92,127]
Encapsulation technique (droplet-based approaches)				
Microfluidic streak plate Grow of single cells in nL droplets; streaking by hand or robotically with a special spindle motor	Has yet to be addressed; might be possible by integrating existing technologies	Inert carrier oil suitable for containment of cells in water microdroplets but not to embed a co-cultivation partner	Limited. Mostly done to grow/screen uncultured species	[105]
SplitChip 1000 microcompartments with two juxtaposed wells for single cell inoculation and splitting for separate analysis of replica cultures	Miniaturized version of replica plating allowing differential analysis/downstream handling of split compartments	Splitting might facilitate downstream analysis, but not the design of multi-partite co-cultivation experiments	High, suitable for single-cell metabolomics	[104]
Gel microdroplets or "nanocultures" Encapsulation of single cells into agarose-based droplets, water-oil-water emulsions or polydimethylsiloxane (PDMS), e.g. for cultivation in percolating columns	Both physicochemical and chemical contacts possible; high surface area-to-volume ratio facilitates diffusion; water-permeable microniches allow control of volume compartment, e.g. by osmosis	Fragility of emulsion droplets and limited understanding of mass transfer hinder long term studies; chemical nature of substances (polarity, size) might be an issue; only spheroid geometries	Very high. Isolation of droplet and metabolome analysis should be possible	[99,101,103]
Other devices/techniques				
Soil chambers, e.g. iChip	Natural environment is used as	*In situ* cultivation might dictate	Limited. Not envisioned by the	[71,84]

Microorganisms are re-implanted into their original environment and grown *in situ*	stimulus to grow the "microbial dark matter" (diffusion of chemicals)	conditions and limit the controlled introduction of further species/strain; size of compartments too small for multispecies consortia	method, but instead used to grow new species	
Hollow-fibre membrane chamber Counterpart of the iChip, but more technical challenging; flux of fluid can be controlled better	Since fluids can be better controlled, supernatants of different cells can be screened in parallel	No cell-cell contacts present	Similar as above	[128]
Low-volume migration chamber Allow *in vivo* neutrophil migration study in zebrafish; imaging possible (microscopy); ports for loading and removal of media and wastes	Designed to dissect function of secondary metabolites ("function-omic" platform); arrayed chambers with automation possible	Performed with purified chemicals; mixing of both media through migration channels (dissipation of gradients unless media is constantly removed/re-filling)	Likely high, but still need to be demonstrated for living cells; especially useful to investigate chemoattraction	[119]
Open microscale platform Open platform for co-cultivation and metabolomic analysis	Integrated liquid-liquid extraction protocol; open nature of the device (liquid-air interface) particularly suitable for downstream analyses; geometry of microchambers is taken into consideration	Co-culture design intended for bi-partite co-cultures; flow of media is limited or done by pipetting (static cultivation conditions); no automation	High. The device was developed with the purpose of performing metabolomics analysis and co-cultivation experiments; multi-partite interactions still based on educated guesses	[110]
3D printing Printing of different geometries (adjacent, nested, free-floating colonies) with laser-based lithographic technique with gelatin	Diffusion of chemical possible; gelatin is porous and biocompatible; high-versatility in defining an exact 3D structure of microbial communities	Rational design of 3D structure required; immobilization of cells might represent a less dynamic situation than that of biofilms in nature; costs relatively high	High. Especially interesting to study the spatial structures of complex multispecies communities	[107]

Box 1. On past and present trends in the (co-)cultivation of microorganisms

Microbiological tools have evolved arguably not as much as tools in other technical fields. After the first cultivation of pure colonies of bacteria by Robert Koch (1843-1910) around 1880 [17,129], axenic growth of strains either on solid or in liquid media belongs to the standard and irreplaceable routine in microbiology. This is dictated by practical reasons, yet microorganisms are alienated from their natural environment, which is characterized by complex, inhomogeneous substrata and promiscuous associations of microorganisms (per some estimates, one gram of soil harbors between 10,000-50,000 different species [130]).

Plating experiments yield only a fraction of the cells observed under the microscope, a phenomenon known since many decades as the “Great Plate Count Anomaly” [131]. Molecular techniques, in particular metagenomics and fluorescent *in situ* hybridisation (FISH), uncovered how these cells are not remnants of dead microorganisms but alive and well – and extremely diverse, representing an estimate 99,9% of all microorganisms [132]. Borrowing a terminology from astrophysics, this wealth of “uncultivable” diversity is referred as the “microbial dark matter” [133,134]. Efforts focusing on growing the seemingly inaccessible microbial wealth from sites as diverse as the human microbiome [135] or soil habitats [71,84] often foresee the integration of different approaches or new microbiology tools.

Given the importance of microbial communication in the production of secondary metabolites, microorganisms represent a treasure chest for natural product discovery. This is illustrated by the tremendous momentum that co-cultivation studies are currently gaining (Figure 1), with the first reported study on “mixed cultures” dating back to 1918 [136]. The author analyzed co-cultures of *E. coli* and *Bacillus paratyphosus* and concluded that *“it is hoped that by these investigations material of particular interest relating to the biochemical and physiological processes within the bacterial culture will be obtained.”* [136] One century later, researchers in the fields of microbiology, biotechnology and natural product discovery still explore co-cultivation experiments as one way to pursue these questions. Given the multiple names given to these studies by the community of microbiologists, we propose to use a unique nomenclature to unify different fields of microbiology by always including the terms “co-cultivation”, “co-cultures” (hyphenated) or “mixed cultures” in the abstract or keywords.

A survey revealed that more than 20,000 natural products with antimicrobial activity from microorganisms have been discovered [137], with around two-thirds of all therapeutically-used antimicrobials like tetracyclines, aminoglycosides, chloramphenicol, macrolides, and glycopeptides coming from actinobacteria and members of the genus *Streptomyces* as undisputed monopolizers [5,6,11]. When filamentous fungi (producing substances like penicillins and cephalosporins) and non-filamentous bacteria (e.g. myxobacteria, *Pseudomonas* spp.) are included, this value reaches 80-90%; among the remaining substances, many are semi-synthetical (i.e. derivatives of natural products) [3]. Despite the extremely specific action of antibiotics and the huge advances in pharmacology since the introduction of the "magic bullet" concept by Paul Ehrlich (1854-1915) to describe chemotherapeutic agents, antibiotics are still used rather unspecifically and at high dosage.

Box 2. On the ways to activate the microbial secondary metabolism and its link with the primary metabolism

In Streptomycetes, both the carbon and the amino sugar metabolism influence antibiotic production [138]. Rigali *et al.* [139] showed that monomeric *N*-acetylglucosamine (GlcNAc) added exogenously on minimal media curbed production of the polyketide (PK) actinorhodin in several *Streptomyces* spp. Importantly, the authors provided convincing evidences for a link between nutritional status, developmental stage and activation of the secondary metabolism. Giving the ubiquitous presence of the amino sugar GlcNAc and its homopolymeric form chitin in nature in the cell wall of fungi, the exoskeleton of insects and the extracellular matrix of mammals (hyaluronic acid) or in its heteropolymeric from in the cell wall of bacteria (murein), this finding raises interesting implications for the influence of exogenous sugar monomers on antibiotic production upon multi-species interactions. During a chemical screening with over 30,000 small molecules to identify conditions inducing antibiotic production in actinomycetes, Craney *et al.* [140] observed more pigmentation (among other due to the increased production of the antibiotics actinorhodin and germicidins) by *S. coelicolor* upon addition of "ARCs" (**a**ntibiotic-**r**emodelling **c**ompounds). These small chemicals showed structural similarities and comparable activities with inhibitors of fatty acid (FA) biosynthesis. Both FA and PK synthesis requires the ubiquitous precursors acetyl-CoA and malonyl-CoA, thus linking primary and secondary metabolism. Partial inhibition of FA synthesis resulted

in a physiological imbalance and an increased availability of substrates for the synthesis of secondary metabolites. These and other studies highlight how the term "secondary metabolism" might be misleading, and in light of the multiple functions that it exerts in nature, it has been proposed to refer to it as a "specialized metabolism" [141].

Crucially, these insights reveal ways to activate the microbial secondary metabolism (Table I) and unlock the potential hidden in the biosynthetic dark matter. [16,35,142,143]. These are divided into knowledge-based and general approaches. For the former, the availability of suitable production hosts and/or genetic engineering tools is a prerequisite for the (heterologous) expression of BGCs or specific transcriptions factors (e.g. [37,144]). Co-cultivation experiments fall into the latter category and can be done without extensive knowledge of the strains used [2,6]. Further general approaches for "genome mining" rely on epigenetic factors involved in chromatin remodeling or global gene expression, either by using mutants or by adding exogenously chemical elicitors like valproic acid, 5-azacytidine or suberoylanilide hydroxamic acid [41–43,45–47,145,146]; on the exogenous addition of chemicals like GlcNAc, cAMP, FA synthesis inhibitors, antibiotics or quorum sensing molecules [29,81,139,140,147,148], rare earth elements like scandium [149]; or on the variations in the abiotic growth conditions ("OSMAC" approach [48]).

Table I. Ways to activate the silent secondary metabolism of microorganisms.

Approach	Comments	Potential for screenings	Selected ref.
Targeted-expression of a given BGC	Requires prior knowledge of the BGC and is often pathway specific (e.g. promoter swapping or heterologous cluster expression)	Very low	[37,144]
Chemical amendments (e.g. antibiotics, GlcNAc, chromatin modifiers, quorum sensing molecules)	Chemicals might be expensive or their bioavailability (e.g. diffusion in medium) might be low; screenings often based on phenotypic readouts (e.g. pigment formation)	High	[42,81,139,140]
Modification of growth or medium conditions (e.g. OSMAC approach)	Parallel experiments under different abiotic condition might be time-consuming	Medium	[48]
Use of mutants (e.g. developmental or	Broad effects on secondary metabolism; might be used in combination with other	Medium (strain specific) Might be high e.g. with transposon mutagenesis	[47,145,146]

epigenetic)	approaches		
Co-cultivation experiments	Often based on educated guesses or serendipitous discoveries of specific interactions; mixed culture experiments with three or more stains/species very rare	Currently very limited. Might be greatly increased by the implementation of existing microbiology tools	[50,51,81,150]

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