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Review Article

Safety aspects of microorganisms deliberately released into the environment

Alexandra Lensch [a], Hanna Abbas Lindfors [b], Elke Duwenig [c], Tobias Fleischmann [d], Carsten Hjort [e], Sirpa O. Kärenlampi [f], Lucie McMurtry [g,*], Emily-Denise Melton [h], Mikael Rørdam Andersen [e], Ryan Skinner [i], Markus Wyss [j], Richard van Kranenburg [k]

[a] *Evonik Operations GmbH, Nutrition & Care, 63457 Hanau-Wolfgang, Germany*
[b] *DSM Food Specialties B.V., 2613 AX, Delft, the Netherlands*
[c] *BASF SE, Nutrition & Health, 67056 Ludwigshafen, Germany*
[d] *Pfizer Pharma GmbH, 10785 Berlin, Germany*
[e] *Novozymes, 2880 Bagsvaerd, Denmark*
[f] *Biosafe - Biological Safety Solutions Ltd., 70210 Kuopio, Finland*
[g] *EuropaBio – The European Association for Bioindustries, 1000 Brussels, Belgium*
[h] *IFF, 2342 BH, Oegstgeest, the Netherlands*
[i] *Lallemand, Lebanon, NH 03766, United States*
[j] *DSM Nutritional Products Ltd., 4303 Kaiseraugst, Switzerland*
[k] *Corbion, 4206 AC, Gorinchem, the Netherlands*

A B S T R A C T

Microorganisms are used in a variety of sectors, including food and feed production, agricultural or environmental applications, and can be optimized for efficacy, safety and sustainability using modern biotechnology techniques. In the EU, genetically modified microorganisms (GMMs) are typically used as production organisms for food and feed products, including enzymes, amino acids, vitamins, flavourings, and oligosaccharides under the Contained Use Directive. Applications involving live GMMs would fall under the Deliberate Release Directive. However, its relevant regulations and guidelines are so far not supporting the access to market of GMMs. In this literature review, we examine what is already known or can be inferred about the safety of microorganisms deliberately released into the environment across sectors. We conclude that there is sufficient evidence supporting the establishment of a European framework for the risk assessment of GMMs deliberately released into the environment, which would enable timely market access for live GMM products.

1. Introduction

Microorganisms have been used safely for decades in biotechnological applications, such as the production of enzymes, oligosaccharides, organic acids, amino acids, vitamins and flavourings, which are commercialized in many different sectors such as food and feed, personal care, and pharmaceuticals. The microorganisms are cultivated in closed facilities (contained use) and are not present in viable form in the final product. Live microorganisms have also been used for centuries in the production of foods such as cheese and bread, beverages such as beer and wine, and as probiotics. Microorganisms used in biotechnological processes have relatively simple, fully resolved and characterized genomes. With modern biotechnology becoming increasingly more sophisticated, new genomic techniques (NGTs[1]), in particular CRISPR-Cas[2] (Stovicek et al., 2017), provide opportunities to improve microorganisms as products or production organisms with unprecedented precision. The resulting microorganisms are increasingly optimized for safety and sustainability. Examples include the removal of genes with potential safety concern, or metabolic engineering to increase (a) the yield of a molecule or process of interest and (b) the nutrient use efficiency, thereby reducing the environmental footprint. These new technologies hold great promise, especially for live microbial products (i.e.,

* Corresponding author.
E-mail address: l.mcmurtry@europabio.org (L. McMurtry).

[1] NGTs are a subset of the gene technology/genetic engineering toolbox; they are defined by the EU Commission as techniques capable of changing or altering the genetic material of an organism and that have emerged or have been developed since 2001, when the existing Deliberate Release Directive 2001/18/EC entered into force [it was last updated on 27 March 2021].

[2] Clustered Regularly Interspaced Short Palindromic Repeats, associated with a cleaving protein (e.g., CRISPR-associated protein 9).

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deliberate release applications).

The current process-based EU regulatory framework for biotechnology products under the Deliberate Release Directive 2001/18/EC and the Contained Use Directive 2009/41/EC needs to be revised in the light of scientific and technological advancements, such as NGTs. A science-based, functional authorisation system is needed to allow timely market access for new innovative and safe products while avoiding implementation challenges, legal uncertainties and distortions of competition and global trade. This already became clear in the European Commission 2021 study on NGTs,[3] which concluded that the GMO legislation is no longer fit for purpose. As a result, a policy action on plants derived from targeted mutagenesis and cisgenesis was initiated. Although the extensive use of NGTs in contained use applications was recognised, the application of NGTs in microorganisms for deliberate release was put on hold on the basis that scientific and safety knowledge was considered limited or lacking. Of particular concern with the deliberate release of microbes into the environment was their self-replicating ability and, consequently, the difficulty in controlling potential adverse effects by stopping further dissemination and removal from the environment.

Here we (a) summarize the current knowledge on the safety of microorganisms deliberately released into the environment; and (b) highlight the benefits of NGTs and other genetic engineering technologies when applied to microorganisms. We consider routes of environmental exposure and risk assessment, detection and control methods, including biocontainment strategies. We also provide an overview of microbial products in deliberate release applications – already existing or in the research/development pipeline – and their environmental risk assessment (ERA). The design of the regulatory landscape to accommodate and encourage the safe development and commercialization of genetically modified microorganisms (GMMs) using genetic engineering techniques will play a crucial role in contributing to the successful realization of the EU Green Deal,[4] including the Farm to Fork strategy[5] and broader sustainability ambitions. The importance of a supportive regulatory framework for the deliberate release of GMMs to attract and incentivize innovation in this area should not be underestimated. The industry will not invest in this field if there is no meaningful way for market access, independently of the vast benefits it might bring. In addition, to keep pace with the fast scientific and technological developments in this field, it would be preferable for a future regulatory framework to focus on product-based rather than process-based assessments. We show that there is sufficient relevant scientific knowledge and safety data available to support the safety assessment of microorganisms obtained by genetic engineering techniques and open the EU market for deliberate release applications of GMMs.

2. What is already known about the safety of microorganisms deliberately released into the environment?

In this section, the current knowledge about the deliberate release of microorganisms into the environment is summarized. The focus is especially on food and feed products containing viable microorganisms – non-GMMs and GMMs. The environment is defined as a person, animal, or plant, and the surroundings (soil, water and air) in which a person, animal, or plant lives or operates.[6]

2.1. Possible routes into the environment

For thousands of years, microorganisms have played an important role in food and feed production. Examples are beverages such as beer and wine, dairy products such as yoghurt, kefir and cheese, as well as probiotics and silage additives for animals. Environmental dissemination of live microorganisms can occur via the intake of food and feed as well as through the sewage system or solid waste disposal.

During consumption, the microorganism enters the human or animal gastrointestinal (GI) tract. The vertebrate GI tract is a hostile environment (e.g., (Goo et al., 2010)). The stomach with its low pH value inactivates many microorganisms. The small and large intestines are the most densely and diversely colonized organs in the human body. Approximately 90% of the microbial population consists of *Firmicutes* and *Bacteroides,* the remaining 10% include *Actinobacteria, Proteobacteria, Fusobacteria,* and *Verrucomicrobia* (Arumugam et al., 2011). On excretion from the human body, faecal microbes travel through the sewage system to wastewater treatment plants where human microbiome-associated species can be found (Cai et al., 2014; Newton et al., 2022). Although the wastewater treatment plant is designed to remove microorganisms (90% of the microorganisms present in wastewater are removed from the final effluent), there is a constant flux of microorganisms from the treated effluent to the surrounding aquatic environment (Mao et al., 2015). Other major routes for microorganisms from urban areas into aquatic environments are discharge from failing water infrastructure and stormwater runoff (Newton and McClary, 2019). Furthermore, aerosolization of surface waters can lead to microbial transport through the atmosphere and redeposition elsewhere in aquatic or terrestrial environments (Lighthart and Stetzenbach, 1994). Thus, if a microorganism added purposefully to food/feed would survive the GI tract and the sewage system and the physico-chemical wastewater treatment process, it is theoretically possible that such microorganism could be deposited or aerosolized in the environment (see also Section *1.2.1 Survival in the environment*).

If the microorganism in a food/feed product is disposed of in solid waste, it can end up in municipal landfill areas. These landfills constitute the third largest anthropogenic source of bioaerosols (Zhang et al., 2023). Such bioaerosols can contain bacterial and fungal species, which are able to cling onto particulate matter (Nair, 2021; Zhang et al., 2023).

2.2. Environmental risk assessment

A considerable amount of research has been and is being carried out to improve microbial strains for feed and food applications. Conventional methods have been used since the 1930s, gene technology since the early 1980s, and new genomic techniques since the 2000s. The goal is to provide efficiency and sustainability benefits, e.g., by improved performance, elimination of genes of potential safety concern, better nutrient use efficiency, and reduction of the environmental footprint.

For feed and food products consisting of, or containing, viable

[3] EU Commission, "Study on the status of new genomic techniques under Union law and in light of the Court of Justice ruling in Case C-528/16" SWD (2021) 92 final. https://food.ec.europa.eu/plants/genetically-modified-organisms/new-techniques-biotechnology/ec-study-new-genomic-techniques_en (accessed 4 December 2023).

[4] EU Commission, The European Green Deal. https://commission.europa.eu/strategy-and-policy/priorities-2019-2024/european-green-deal_en (accessed 16 January 2024).

[5] "*New innovative techniques, including biotechnology and the development of bio-based products, may play a role in increasing sustainability*", p. 8, EU Commission, "A Farm to Fork Strategy for a fair, healthy and environmentally-friendly food system" (Communication), COM(2020) 381 final. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52020DC0381 (accessed 16 January 2024).

[6] According to Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, 'environmental risk assessment' means the evaluation of risks to human health and the environment, whether direct or indirect, immediate or delayed, which the deliberate release or the placing on the market of GMOs may pose.Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC, (2001), Official Journal L 106, p. 1. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32001L0018 (accessed 4 December 2023).

microorganisms, including GMMs, it may be necessary to assess the impact of major changes in the biology of the microorganism, in terms of potential adverse or beneficial effects on the consumer (toxicity, pathogenicity, significant effects on the gut microbiota), and on the environment (persistence and significant effects on the ecosystem) (Aguilera et al., 2013). The environmental risk assessment (ERA) may consider whether the GMM survives and persists in the environment (including the human/animal GI tract) better than the non-GMM counterpart, and whether it eventually multiplies and/or mates with indigenous microorganisms. The consequences of potential transfer of genes of concern, like antimicrobial resistance genes, are also considered (ILSI Europe, 1999). The concerns with regard to the survival of microorganisms in the environment and antimicrobial resistance (AMR) and its spread are considered in more detail below.

2.2.1. Survival in the environment

Microbes play a key role in the functioning of ecosystems. They are key drivers of a wide range of ecosystem services including soil nutrient cycling, plant growth promotion, marine biogeochemical processes, and maintenance of human and animal health. Importantly, microbial communities harbor key taxa that significantly influence the community composition and microbiome performance, irrespective of their abundance (Banerjee et al., 2018).

Whether or not a microorganism introduced in the environment can become established and persistent will depend primarily on its fitness in a particular environment (Lenski, 1993). Because permutations in the environment are practically limitless, it is not possible to test the fitness empirically in contained environments (De Leij et al., 1998). Consequently, the lack of empirical data on the environmental fitness of microorganisms (non-GM and GM) has led to overly restrictive legislative measures that are designed to safeguard against the risks associated with their release.

It is commonly assumed that microorganisms modified by modern molecular methods are unfit to survive and multiply in nature (Lenski, 1993; Maull and Solé, 2022). The reason for such microorganisms to be poor competitors would be energetic inefficiency, disruption of genomic coadaptation, or domestication. Davies stated that “recombinant microorganisms are essentially non-competitive and are unlikely to have much chance of establishing themselves in highly competitive natural environments” (Davies, 1988). According to Brill, randomly introduced microorganisms generally are unable to predominate in new habitats because preexisting organisms already have evolved to successfully compete for those niches (Brill, 1985). In most cases, a microbe multiplies far more slowly in nature than in the laboratory. The extra burden of carrying new genes would often decrease the ability to compete and persist.

Based on well-established principles of evolutionary biology and microbiology, Davis concluded that (a) deliberate introduction of large amounts of GMMs into the environment is not substantially more dangerous than the accidental release of small amounts, which is currently of concern; (b) distant organisms are less likely to yield dangerous hybrids than more closely related ones; and (c) complex attributes of pathogenicity are not likely to emerge from genetic alterations in nonpathogens (Davis, 1987).

Moreover, experiments using mixtures of GMMs and wild-type microorganisms often show that GMMs are less fit than the wild-type organisms from which they are derived (De Leij et al., 1998). In some cases, however, no effects and even enhanced survival of a GMM have been reported (De Leij et al., 1998). In one of these cases, an insertion sequence, which encodes a transposase and an inhibitor of transposition of the kanamycin-resistance transposon Tn5, increased the growth rate of *Escherichia coli* K-12 cells under competition in continuous culture (Hartl et al., 1983). However, this was established under chemostat conditions, which offer a limited comparison to complex environmental ecosystems (Smith et al., 1992).

The release of bacteria genetically marked to permit their monitoring after inoculation to plants in field ecosystems has allowed a better understanding of the interaction between the GMMs and the ecosystem (Amarger, 2002). Bacteria genetically modified to be easily traceable and/or improved in the expression of beneficial traits have been released to plants in several experimental field plots. Local environmental conditions appeared as the main factors determining survival and persistence of the bacteria and in the expression of beneficial traits. The spread of inoculant bacteria from their point of dissemination was limited. Transient shifts in favor of the released bacteria and in disfavor of some members of the bacterial and fungal populations present in the plant rhizosphere might occur with certain released bacteria. The changes observed were, however, less important than those observed under usual agricultural practices. Gene transfer from the resident population to introduced bacteria was detected in one case. The transconjugants were found only transiently in the phytosphere of plants but not in the soil. No differences in survival, spread, persistence in the field and ecological impacts were detected between genetically modified bacteria and the corresponding unmodified parental strain (Amarger, 2002).

Maull and Solé addressed the issue of ecological impact and possible diversity losses by modelling the response of a community to the addition of a synthetic strain derived from a member of a stable ecosystem (Maull and Solé, 2022). They showed that interactions in the established community largely limit the spread of the engineered strain, thus suggesting that species diversity acts as an ecological firewall.

Moreover, the genetic basis of domestication of microbes is becoming better understood. Specific SNPs (single nucleotide polymorphisms), copy number variations, chromosomal rearrangements, genomic decay and other mechanisms can make microbes more fit for the manufacturing environment but less fit for natural environments (Steensels et al., 2019).

2.2.2. Antimicrobial resistance and its spread

The origins and transfer routes of antimicrobial resistance (AMR) are complex. Resistant bacteria and associated AMR genes in food-producing environments can originate from indigenous environmental bacteria or be introduced by humans or livestock through animal and human waste streams such as manure, sewage sludge or water contaminated with faecal matter. In addition, transmission between livestock and between wild animals and livestock will also affect the spread of AMR within livestock and food-producing environmental microbiomes (Koutsoumanis et al., 2021).

2.2.3. Clinical relevance

In the pre-antibiotic era, contagious microbial diseases such as smallpox, cholera, diphtheria, pneumonia, typhoid fever, plague, tuberculosis, typhus and syphilis were widespread, and infectious diseases were the primary cause of morbidity and mortality (Dhingra et al., 2020).

With the discovery of penicillin by Alexander Fleming in 1928, the human health benefits were immense. In the meantime, antibiotics interfering with almost every process in the bacterial cell are known. Based on their structure and mode of action, at least seven major groups of antibiotics have been described. These include β-lactams and glycopeptides (process affected: cell wall synthesis); aminoglycosides, macrolides and tetracyclines (process affected: protein synthesis); daptomycin (process affected: cell membrane function); and platensimycin (process affected: fatty acid biosynthesis) (Peterson and Kaur, 2018).

The success in treating infectious diseases started to fade when pathogenic microorganisms acquired resistance to the antibiotics, mainly due to the selection pressure during treatment. Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), resistant *Clostridioides difficile,* multidrug-resistant *Pseudomonas aeruginosa* and carbapenem-resistant *Acinetobacter baumannii* are some of the bacteria that pose serious public health challenges

worldwide (Bartal et al., 2022; Dhingra et al., 2020).

Antibiotic resistant bacteria are not confined to the clinic. Soil bacteria are abundant sources of both antimicrobials and resistance genes, and the majority of clinical antibiotics are actually derived from microbial soil products. Bacteria that produce antimicrobials need to have mechanisms to protect themselves from their own antibiotics. However, unlike clinical microorganisms, antibiotic-producing environmental microorganisms demonstrate a significant degree of intrinsic resistance that appears to be independent of the selective pressure – an attribute that has been suggested to be ancient, existing long before clinical antibiotic uses (Singer et al., 2016).

Intrinsic and acquired resistance

The resistance mechanisms can be innate to the microorganism (= intrinsic resistance) or transferred from other microorganisms (= acquired resistance) (Dhingra et al., 2020). Intrinsic resistance suggests it is found in the majority of the genomes of the bacterial species, while acquired resistance suggests it could be acquired by means of new genetic material or through sporadic mutations of intrinsic genes (Singer et al., 2016). Intrinsic resistance in bacteria is often a consequence of changes in cell envelope permeability and activity of efflux pumps (Ramakrishna et al., 2019).

2.2.4. Resistance mechanisms

The principal mechanisms of antimicrobial resistance are the modification or degradation of the antimicrobial, its active removal (efflux) from the cells, its sequestration by special proteins, and the modification, bypass or protection of an antibiotic target (Darby et al., 2023; Peterson and Kaur, 2018). Structural modification is a common strategy for rendering an antibiotic ineffective, especially in the case of aminoglycoside antibiotics (for example, kanamycin, gentamycin and streptomycin), chloramphenicol, and β-lactams. A high number of aminoglycoside-modifying enzymes is known that acetylate, phosphorylate, or adenylylate the antibiotics. The antibiotic efflux strategy includes ABC (ATP binding cassette) transporters or proton gradients. Antibiotic sequestration involves drug-binding proteins, which prevent the antibiotic from reaching its target. Target modification acts as a self-resistance mechanism against several classes of antibiotics, including β-lactams, glycopeptides, macrolides, lincosamides, streptogramins, and aminoglycosides. The antibiotic target bypass mechanism is based on additional low-affinity targets. In case of an antibiotic target protection the antibiotic is removed from the target site.

2.2.5. Horizontal gene transfer

Horizontal gene transfer (HGT) among microorganisms in nature is arguably the most conspicuous feature of bacterial evolution (Arnold et al., 2022; Mahdi et al., 2022; Popa and Dagan, 2011). Evidence for HGT is found in most bacterial genomes.

In microbiomes, HGT is an evolutionary mechanism for the development of the consortium of microorganisms. Different parts of the genomes of the organisms are particularly active in HGT. These are termed as the mobilome (Brito, 2021). Among the genes conferring diverse functions that provide increased fitness, antimicrobial resistance genes are relevant for safety and are therefore considered here in particular.

A major mechanism for acquiring resistance is through mobile genetic elements (MGEs). Genetic elements can be transferred between cells through: (1) transformation, (2) conjugation, (3) transduction or (4) vesiduction. Transformation is the acquisition of naked DNA from the environment. Conjugation is the transfer of genes through direct contact between two bacteria. Transduction is the transfer of genes from bacteriophage to bacteria during infection of a bacterial cell. In vesiduction, the DNA is packaged into lipid membrane vesicles before the transfer (Soler and Forterre, 2020).

The risk of antimicrobial resistance being transferred from environmental microorganisms to pathogens is already apparent and of significant concern globally (Singer et al., 2016). In a recent study, lactic acid bacteria and Bifidobacteria were analyzed for their phenotypic susceptibility to antimicrobials. The AMR genes and MGEs were characterized in the genomes of 1114 strains using comparative genomics (Rozman et al., 2022). Phenotypic susceptibility data showed that antimicrobial resistance was more common in the intestinal isolates than in the commercial strains. Intrinsic AMR genes were abundant in Enterococci, Bifidobacteria, and Lactococci, but were considered non-risky due to the absence of MGEs. Of the commercial strains, 13.8% contained acquired AMR genes. However, AMR genes and MGEs were not as abundant or diverse in the commercial strains as in the human intestinal isolates or the isolates from human milk. For this reason, the authors suggested that strains intentionally introduced into the agri-food chain do not pose a hazard.

Resistance genes can be spread in mixed-species populations via HGT (Huddleston, 2014; Lerminiaux and Cameron, 2019). However, interspecies HGT is rare, as HGT mainly occurs between closely related strains (De Wit et al., 2022). In addition, segregation in structured environments can further limit gene transfer between competing species. Another interesting recent finding is that short chain fatty acids (aliphatic carboxylic acids composed of 1 to 6 carbon atoms) can inhibit the transfer of plasmids between bacteria in the GI tract (Mellata and Ott, 2023). However, several mechanisms could increase the horizontal transfer of resistance genes in competitive communities. For example, the SOS stress response to DNA damage has been shown to increase the conjugative transfer of AMR genes (Beaber et al., 2004). In this case, integrating conjugative elements (ICEs), a diverse group of MGEs transferred via cell–cell contact and integrated into the chromosome of the new host, play a crucial role in spreading the AMR genes (Beaber et al., 2004). Since the SOS stress response system is also activated by antibiotics and bacteriocins (Andersson and Hughes, 2014; Walker et al., 2004), competition could also enhance the spread of resistance genes via conjugation.

2.2.6. Transfer frequencies

Horizontal gene transfer is best understood in bacteria (Arnold et al., 2022). Transfer frequencies vary greatly depending on the mechanism.

A meta-analysis of antimicrobial resistance gene transfer rates during conjugation reported rates (transfer probability per cell) of 10^{-2} to 10^{-9}, with higher frequencies observed between taxonomically more closely related donor and recipient strains (Hunter et al., 2008). The same association between transfer frequency and taxonomic relatedness was found in a meta-analysis of plasmid conjugation frequencies from *E. coli* donors to various recipient species, but only in liquid broth matings, where conjugation frequencies varied over nine orders of magnitude ($1.0 \times 10^{-9} - 1.3$) (Alderliesten et al., 2020). In contrast, no significant association between relatedness and transfer frequency was observed for filter paper matings, where conjugation frequencies varied over 11 orders of magnitude ($4.5 \times 10^{-11} - 2.1$) (Alderliesten et al., 2020).

In addition to the taxonomic relatedness of donor and recipient organisms and the test method used, HGT frequencies are influenced by a wide range of abiotic and biotic factors. In soil, the moisture, temperature, pH and soil type all affect the HGT frequency, as does the presence of earthworms, protozoa and fungi. In aquatic environments, frequencies have been found to differ between freshwater and marine environments. In vivo environments, such as the mammalian or insect gut, might enhance the transfer frequency, as may biofilm environments (Aminov, 2011).

2.2.7. Selection pressure

The dissemination and maintenance of the resistance genes is also influenced by the selection pressure. It is recognized that the use of antimicrobials, or certain biocides and metals, are important factors in the occurrence and further selection and spread of antimicrobial resistance (Koutsoumanis et al., 2021).

Selection occurs when a single resistant bacterium in a population is provided with the opportunity to become more prevalent because of

killing or suppression of the previously dominant susceptible population (Murphy et al., 2017). Such opportunity can result from the application of an antimicrobial to which the organism exhibits reduced susceptibility or clinical resistance. The single resistant bacterium then multiplies, often in an exponential way, until a new equilibrium is reached, where the resistant bacterium becomes more dominant within the population (Baquero, 2011).

In the treated host, the selection process is driven by the drug pharmacokinetics and dosage regimen. The dosage regimen is defined by the dosage (mg/kg), the route of administration (formulation), the treatment interval and the treatment duration (Murphy et al., 2017). The selection pressure in the environment will depend on the concentration of antimicrobials in the animal faeces, in the treated crop or aquaculture, or in the environmental sources entering the food production systems (e.g., antimicrobial-contaminated water entering the aquaculture system), and for how long these antimicrobial residues persist in the environment (Koutsoumanis et al., 2021).

2.2.8. Dissemination

Several genetic and environmental factors interact in the selection and dissemination of antimicrobial resistance in bacteria. In antimicrobial therapy, the principal bacterial factors are the resistance determinants and the genetic context of the resistance determinants within the bacterial genome (ECDC/EFSA/EMA, 2015).

The social life of bacteria is one of the strongest determinants for their behavior (Burmølle et al., 2014). Most microbial communities, whether on the skin, in the GI tract, in soil, or on various surfaces, are highly diverse and heterogeneous (De Wit et al., 2022). Social interactions, i.e., competition and cooperation between the species, can strongly influence the development and spread of antimicrobial resistance. Cooperation is common between members of the same species due to their high genetic relatedness. Competitive interactions dominate in mixed-species communities. An interesting example of social interactions within microbial communities affecting the emergence and spread of AMR is that probiotic treatment alone decreased the number of AMR genes in the gut resistome, while a combination of probiotics and antibiotics expanded the intestinal resistome more than did the antibiotic treatment in the absence of probiotics (Das et al., 2020).

It is believed that co-existence of antibiotic producer and non-producer organisms has resulted in co-evolution of resistance mechanisms in non-producing environmental bacteria (Das et al., 2020). Resistance determinants found in these two groups of bacteria have gained significant attention in recent years because of their possible link with the emergence of resistance in pathogenic clinical isolates (Peterson and Kaur, 2018).

In the absence of antibiotic pressure, several resistance mechanisms result in a reduced growth rate (Melnyk et al., 2015). However, some resistance-conferring mutations do not result in a significant cost, being selection neutral (De Wit et al., 2022; Koutsoumanis et al., 2021). Competition for resources or space can amplify the fitness cost of resistance mechanisms, limiting the fitness advantage of resistance. At subinhibitory antibiotic concentrations, the fitness advantage of harboring resistance mechanisms can be limited because of competition for the resources (De Wit et al., 2022; Klümper et al., 2019; Koutsoumanis et al., 2021).

Competition can thus counteract the spread of resistance, but it can also contribute to the spread of resistance. At high antibiotic concentrations, antibiotic resistant strains are expected to be competitive regardless of the cost of the resistance mechanism or the composition of the community being exposed.

Perhaps more unexpected is that competitive interactions can lead to the selection of resistance phenotypes even in the absence of clinical antibiotics (De Wit et al., 2022). Indeed, antibiotic resistant bacteria are found in environments not exposed to clinical antibiotics. A plausible explanation is that the antibiotic-producing microorganisms in the environment harbor mechanisms both for self-resistance and resistance to antibiotics produced by the competitors.

The above cases emphasize that gene transfer frequencies alone are of little value in predicting the outcomes. Importantly, the relative contributions of various mechanisms are case-specific. The outcome depends on the exposed microbial community, resource availability, resistance mechanism, and the presence of antibiotics.

2.2.9. Relevance for GMMs deliberately released into the environment

The likelihood of the survival of GMMs in the environment can be summarized as follows:

- Field tests of the release of GMMs to the soil demonstrate that the change in the existing microbiome is transient and less significant than the changes caused by other agricultural practices.
- GMMs are typically poor competitors and therefore unable to persist in the wild. Possible explanations for this loss of competitive fitness include energetic inefficiency, disruption of genomic coadaptation, and domestication.
- Any environmental effects are likely to be more persistent if the microorganism establishes a self-sustaining population. Whether or not a GMM persists depends primarily on its competitive fitness relative to its wild-type counterparts.

Humans are exposed to pathogenic bacteria, antibiotic-resistant bacteria, and AMR genes through several major pathways, including human and veterinary medicine, agriculture, and food consumption (Chen et al., 2019; Mahdi et al., 2022).

Genetic factors influencing the spread of AMR organisms and genes therein from animals to humans and vice-versa include the integration of resistance genes into horizontally transferable genetic elements (e.g., conjugative or mobilizable plasmids, transposons, integron-gene cassettes), and the presence of a cluster of linked AMR or other selectable genes facilitating co-selection of resistance to one substance during exposure to an unrelated substance.

The gut microbiota is regarded as the largest reservoir of transmissible antimicrobial resistance genes, not only within livestock but also in humans. Such bacteria can act as a donor, vector or recipient of AMR genes. As a recent study on lactic acid bacteria and Bifidobacteria shows, the phenotypic resistance to antibiotics, AMR genes and mobile genetic elements are more common in intestinal isolates than in commercial strains (Rozman et al., 2022). This suggests that strains intentionally introduced into the agri-food chain do not pose a marked threat, as they do not significantly increase the antimicrobial resistance gene pool. However, attention should be paid especially to individual probiotic strains carrying AMR genes and containing genetic elements that have been shown to have a high potential for transfer in the gut microbiota.

It can be concluded that most engineered microbes do not need to be regulated more strictly than non-engineered microbial strains that have been tested in the field in the past. Exceptions would be strains derived from microbial species that are pathogenic or toxic for humans and animals and/or which include genes derived from pathogenic organisms. Moreover, microbial strains intended for use as food or feed should not contribute further to the reservoir of antibiotic resistance genes already present in the gut flora of humans and animals or in the environment. The efficiency of the methods to modify microorganisms in a targeted manner improved over time, and the use of antibiotic resistance genes as selection markers is either no longer required, or they can be easily removed in organisms to be used commercially. State-of-the-art commercial microbial strains do not contain antibiotic resistance markers.

2.3. Detection methods for GMM traceability and control

In general, detection of non-GMMs and GMMs is based on cultivation and/or taking advantage of the specific properties of the microorganism.

To enumerate the microorganism of interest, it is first cultivated on a selective medium that favours its growth. Detection, identification, enumeration and/or monitoring can be based on various principles and techniques; for a recent review see e.g. (Ferone et al., 2020):

- conventional microbiological methods, e.g., morphology (shape, size), staining (Gram), and physiology (API® tests, detection of specific enzymes);
- using antibodies or oligonucleotides coupled to fluorescent dyes to identify a species or a taxonomic group;
- DNA-based methods, e.g., sequencing, polymerase chain reaction (PCR), and Pulsed Field Gel Electrophoresis (PFGE);
- peptide fingerprinting (MALDI-TOF) and other spectroscopic techniques, e.g., near-infrared spectroscopy, Raman spectroscopy, surface enhanced Raman spectroscopy, and hyperspectral imaging;
- secondary metabolite profiling, e.g., for filamentous fungi and Actinomycetes;
- biosensors.

In the case of GMMs, genetic modifications usually introduce specific signatures that can be used for detection. In the EU, Directive 2001/18/EC (Annex III A, Section V. A.) stipulates that a dossier approved for the deliberate release of a GMM needs to contain information on its specific monitoring technique. Methods for GMM detection are typically based on PCR targeting strain-specific DNA markers (Holst-Jensen et al., 2003). The EC Joint Research Centre (JRC) provides guidance and minimum performance requirements for such tests for genetically modified food and feed in the context of Regulation (EC) No 1829/2003 (Marchesi et al., 2015). The European Network of GMO Laboratories (ENGL) assists the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) with validation of the methods. The qPCR methods QL-EVE-EC-001 for the detection of GM *E. coli* K-12 strain AG3139 and QL-EVE-EC-002 for the detection of GM *E. coli* K-12 strain 19E are examples of methods approved by EURL GMFF[7] (Mazzara et al., 2009).

In the 1990s, several studies looked at the persistence of GMMs introduced into the environment. Markers like antibiotic resistance, β-galactosidase, catechol 2,3-dioxygenase and bioluminescence were used for the detection and enumeration of viable cells (Prosser, 1994). Smalla and van Elsas reviewed the sampling and detection methods for soil (Smalla and van Elsas, 1996). Shaw et al. studied a bioluminescent *Xanthomonas campestris* strain applied on cabbage plants and incorporated into the soil (Shaw et al., 1992). Survival of *lux*-marked GM *E. coli* and GM *Bacillus subtilis* in soil and the rhizosphere of bean (Kozdrój, 1996) and containment of *lux*-marked *Pseudomonas fluorescens* sprayed on naphthalene-contaminated soil (Ford et al., 1999) have been monitored. De Leij et al. studied the impact of GM *P. fluorescens,* marked with *lacZY* and kanamycin resistance, on indigenous microbial populations of wheat (De Leij et al., 1995). Morgan et al. looked at the survival of *Xenorhabdus nematophilus* and *Photorhabdus luminescens* that were marked with a kanamycin resistance gene and a *xylE* gene to aid in their detection in water and soil (Morgan et al., 1997). Ferguson et al. monitored the survival of *lux*-marked *Aeromonas salmonicida* in seawater (Ferguson et al., 1995). Yeom et al. successfully applied the most probable number (MPN) method with multiplex PCR and a simpler MPN-DNA dot blot method to detect GM *E. coli, Pseudomonas putida*, and *Acinetobacter oleivorans* harboring antibiotic resistance, *gfp* and *lacZ* genes as markers for the detection in soil (Yeom et al., 2011). Finally, Hassan monitored survival of GM *Pseudomonas aeruginosa* in river water by selective plating (Hassan, 2011).

Taken together, methods for effective tracing of GMMs exist. However, these methods need to be developed and validated for each GMM individually to enable specific and reliable detection, identification and quantification of the GMM of interest.

2.4. Biocontainment strategies

The deliberate release of engineered microbes into the environment is commonly associated with a risk that functions desirable in a target environment are undesirable in other environments. Other possible risks are failure to function in the target environment or acquisition and functional expression of the engineered DNA sequences in other organisms (Pantoja Angles et al., 2022; Parker and Kunjapur, 2020; Pei et al., 2022). However, since the first field trial for evaluating a GM *Pseudomonas syringae* in 1987, academia and industry have investigated GMMs for decades without notable accidents and/or environmental concerns (Ke et al., 2021).

In certain cases, biocontainment can be a solution to address risks by preventing spreading of GMMs in the environment. Strategies for biocontainment are summarized below (Pantoja Angles et al., 2022; Parker and Kunjapur, 2020; Pei et al., 2022):

- Auxotrophy - dependence of the organism on an essential nutrient or metabolite that is not easily found in the environment;
- Transcriptional control or gene circuit - dependence of the organism on a substance required for the expression of housekeeping genes; or
- Toxin–antitoxin system or kill switch - restriction of the organism to a limited environment where the expression of a toxin or cell lysis mechanism is suppressed.

A comprehensive collection of biosafety and biocontainment solutions is available in Biocontainment Finder[8] (Pei et al., 2022). The information includes the main feature and the microorganisms involved, its efficiency (measured or estimated by escape frequency), the tested or proposed application(s), the concern(s) or constraint(s), and the reference to make it easily usable.

One example of a biocontainment system in lactic acid bacteria that is published and accepted by the authorities is the deletion of the *thyA* gene encoding thymidine synthase, rendering the bacteria dependent on thymine supplementation (Plavec and Berlec, 2020). The acceptance of *thyA*-deficient *Lactococcus lactis* as a standard for biological containment is growing worldwide, as it has been approved for human applications by the Belgian Biosafety Advisory Council, Health Canada, the Swedish Medical Products Agency, and the Canadian Environmental Protection Agency (Bahey-El-Din and Gahan, 2010; Steidler et al., 2009).

It is known that engineered pathways allow the use of ecologically rare nitrogen or phosphorus sources such as melamine, cyanamide or phosphite (Shaw et al., 2016). Although this should dramatically reduce the environmental survival of GMMs, it cannot be ruled out that in natural environments these essential metabolites are provided by other microorganisms, or that deleted essential genes are regained via horizontal gene transfer, or that mutations occur disrupting the killing gene (Lee et al., 2018).

A further development is auxotrophies of genomically recoded organisms, in which essential genes depend on synthetic amino acids for expression (Mandell et al., 2015; Rovner et al., 2015), or xeno-nucleic acid (XNA) made from unnatural nucleotides (Lee et al., 2018). These approaches eliminate the risk that essential nutrients are accidentally provided by other microorganisms in the environment or that auxotrophies are restored by horizontal gene transfer. A practical drawback of these biocontainment approaches is that they require costly medium

[7] EURL GMFF Qualitative PCR method for detection of *E. coli* K-12 event 19E. https://gmo-crl.jrc.ec.europa.eu/summaries/CRL-VL-06-08-VR.pdf (accessed 4 December 2023); Qualitative PCR method for detection of *E. coli* K-12 event AG3149 (Mazarra et al., 2009) https://gmo-crl.jrc.ec.europa.eu/gmomethods/docs/QL-EVE-EC-001.pdf (accessed 4 December 2023).

[8] Biocontainment Finder, 2021. https://standardsinsynbio.eu/biocontainment-finder/ (accessed 4 December 2023).

ingredients and can only be economically justified for high-value specialty products rather than for commodity or bulk products. Another possibility to enable recipient-specific and population-level control are cell-to-cell communication systems mediated by signaling molecules or bacteriophages.

Several companies are investigating biocontainment strategies (e.g., Synlogic, Pivot Bio, JOYN Bio, or NOVOME Biotechnologies). Furthermore, several US agencies are developing programs for biosafety, biosecurity, and biocontainment strategies to increase understanding of how synthetic DNA and GMMs behave under various environmental conditions and across diverse organisms for multiple generations[9] (Arnolds et al., 2021).

The above-mentioned tracking, safeguarding, and controlling systems point to a novel direction. In principle, these innovations could offer safe and effective strategies for environmental use of GMMs if needed. However, these systems require further genetic modifications, which will create more challenges under current legislation. Such biocontainment measures should only be required in case of substantiated safety risks.

3. Examples of microbial products used in deliberate release applications and their environmental risk assessments

Although microbes naturally occupy our bodies and surroundings, GMMs are currently almost exclusively restricted to contained use applications in closed vessels and fermenters. However, already for some decades, the deliberate release of GMMs outside of fermenters has been under consideration and scrutiny given the potential benefits in biomedical and environmental areas due to significant innovations in biotechnology, such as advanced genetic engineering and sequencing technologies and the increasing amount of microbial genome data. Whereas many microorganisms are naturally pathogenic, genetic engineering allows the development of safe microorganisms, which perform functions beneficial for humans, animals and the environment.

In this section, we discuss examples of current and foreseeable deliberate release applications of GMMs. We focus on the knowledge that can be gained from the environmental risk assessment of various cases regarding their safety related to the deliberate release into the environment.

3.1. Biomass from fermentation products

The GMMs employed in fermentation processes are typically separated from their target products (e.g., amino acids, vitamins and enzymes) and then recycled or destroyed. Elimination routes include disposal in sanitary landfills, incineration, use as fuel, as animal feed, or reuse as an agricultural soil amendment or other value-added products like in construction material.

Already in 2001, Andersen and colleagues investigated whether antibiotic resistance genes present in inactivated GMM biomass from fermentation, when used as a fertilizer, have an impact on microbial communities in the environment (Andersen et al., 2001). No differences were found in the antibiotic resistance profile of the indigenous bacterial population in the fields treated with the GMM biomass compared with fields treated with inorganic fertilizers. In addition, DNA isolated from the fields for up to 7 years was analyzed with PCR and no production strain-specific genes could be detected.

Halter and Zahn studied the degradation of DNA markers in the heat-inactivated biomass from a GMM used for commercial production of 1,3-propanediol. Laboratory and field tests showed that after two weeks, heterologous DNA from the GMM was no longer detectable with PCR. In addition, there was no evidence for horizontal transfer of DNA from the GMM to organisms present in the soil (Halter and Zahn, 2017).

Coproducts from ethanol production from grain are commonly used in animal feed. These dried distillers' grains (DDGs) contain inactivated microorganisms used in fermentation, typically *Saccharomyces cerevisiae*. Modified versions of *S. cerevisiae* have been used commercially in North American biofuel production since 2012[10] (Retka Schill, 2015), resulting in DDGs containing GM yeast. The Association of American Feed Control Officials has listed definitions (dependent on a favorable safety assessment) for GM yeast used for production of distillers' products.[11] This includes *S. cerevisiae* with modified metabolic and secreted enzyme pathways to improve product yield and reduce the need for inputs of external process enzymes.

3.2. Probiotics

Probiotics are live microorganisms similar to the beneficial microorganisms found in the human or animal gut. They have potential to benefit the health of consumers by maintaining or improving the intestinal microbial flora (Arora and Baldi, 2015). Probiotics have been considered an economical and safe alternative for the treatment of numerous chronic diseases and for the improvement of human and animal health (Yadav et al., 2022). Probiotics are known to modulate host immunity and protect from several infectious and non-infectious diseases. The colonization, killing of pathogens and induction of host cells are a few of the important probiotic attributes which affect several functions of the host (Yadav et al., 2022).

Many probiotic products have been introduced into the international market, for example as food supplements, dietary supplements, natural health products, functional foods, feed additives, and many other categories. Almost without exception, these probiotic products contain wild-type strains. Through genetic engineering, it would be possible not only to strengthen the effects of current strains, but also to develop completely new probiotics (Steidler, 2003). Probiotic products need not necessarily be composed only of bacterial strains but can also include natural compounds in the formulation to induce a particular growth stage or enzymes from external sources. The knowledge of the mechanism by which defined probiotic strains contribute to health allows the rational design of probiotic microorganisms through genetic engineering. If carefully designed and paying specific attention to biological safety, the GM probiotics have the potential to revolutionize alimentary health (Steidler, 2003). Indeed, genetic engineering of microbial probiotic strains is quite promising (see an overview of examples in (Ma et al., 2022)).

In August 2019, it was announced that "the world's first GMO probiotic is for sale"[12] (Cross, 2019). The San Francisco-based start-up ZBiotics launched its new probiotic drink in the US – which from a regulatory perspective is considered a food. Vials were filled with *Bacillus subtilis* ZB183™ that the company had genetically engineered to break down acetaldehyde, which is a molecule that lingers in the body after alcohol is metabolized. In a 90-day repeated-dose oral toxicity study on lyophilized spores, no test item-related clinical effects were

[9] Obama White House, 2015. A National Biosafety and Biosecurity System in the United States. https://obamawhitehouse.archives.gov/blog/2015/10/29/national-biosafety-and-biosecurity-system-united-states (accessed 4 December 2023); DARPA, 2017. Safe Genes Program. https://www.darpa.mil/program/safe-genes (accessed 4 December 2023).

[10] Retka Schill, S. 2015. Yearning for new yeasts. https://ethanolproducer.com/articles/yearning-for-new-yeasts-12004 (accessed 4 December 2023).

[11] AAFCO, 2022. https://apps.fass.org/FASS/survey/content/2022_OP_Chapter_6_enc.pdf#toolbar=0 (accessed 4 December 2023).

[12] Cross, R. 2019. The world's first GMO probiotic is for sale; it's designed to prevent hangovers. https://cen.acs.org/business/start-ups/worlds-first-GMO-probiotic-sale/97/web/2019/08 (accessed 4 December 2023).

observed (Appala Naidu et al., 2019).

Engineered bacteria could also help to protect "good" gut microbes from antibiotics.[13] This is important especially following an antibiotic treatment. Some patients are at risk of developing inflammation or opportunistic infections, e.g., *Clostridioides difficile*. To protect the microbiota from antibiotics, researchers at the Massachusetts Institute of Technology engineered a *Lactococcus lactis* strain, which is normally used in cheese production, to produce an enzyme that breaks down beta-lactam antibiotics (Cubillos-Ruiz et al., 2022). When these bacteria are delivered orally, they transiently populate the intestines, where they secrete the beta-lactamase. This enzyme then breaks down antibiotics that reach the intestinal tract. Consequently, this engineered *L. lactis* weakens the impact of antibiotic treatment on the GI tract by locally degrading the antibiotic and thereby reducing the risk of infections. Beta-lactamase enzymes confer antibiotic resistance to the harboring cells and their genes can readily spread among different bacteria. To address this, the researchers broke up the gene for beta-lactamase into two pieces, each of which encodes a fragment of the enzyme. These gene segments are located on different pieces of DNA, making it very unlikely that both gene segments would be transferred simultaneously to another bacterial cell. This specific biocontainment strategy enables the delivery of antibiotic-degrading enzymes to the gut without the risk of horizontal gene transfer to other bacteria.

The engineering of advanced probiotics is also the core competency of General Probiotics Inc. This company develops live therapeutics to eliminate antibiotic-resistant bacteria and hard-to-treat viruses. They use artificial intelligence and advanced bioengineering techniques to tailor probiotics that effectively target pathogens. Recently, a preliminary approval from the US Food and Drug Administration (FDA) was obtained for a genetically engineered probiotic, which improves feed conversion in poultry and reduces mortality against the bacterium *Clostridium perfringens*, a causal agent of necrotic enteritis[14] (Doughman, 2023).

Live bacterial therapeutics (LBTs) could reverse diseases by establishing themselves in the gut and providing persistent beneficial functions in the host (Russell et al., 2022). A key step in the engineering of LBTs is the selection of the microbial host, or chassis, which would enable sensing of the environment, regulated gene expression, and production of a therapeutic product. Current LBT chassis organisms (e.g., *E. coli* Nissle 1917, *Bacteroides* spp., *Lactobacillus* spp.) cannot establish or even survive in the gut luminal environment (Braat et al., 2006; Puurunen et al., 2021). In a proof-of-concept study, Russell and colleagues used native bacteria as chassis for transgene delivery to impact the physiology of mice (Russell et al., 2022). Native *E. coli* bacteria isolated from the stool cultures of the mice were modified to express functional genes. The reintroduction of these strains induced establishment in the intestine.

In recent years, new probiotic-related concepts such as postbiotics and paraprobiotics have been introduced to describe non-viable microorganisms or cell-free bacterial extracts that may provide benefits to the host by offering various bioactivities (Cuevas-González et al., 2020; Thorakkattu et al., 2022; Vallejo-Cordoba et al., 2020). In vitro and in vivo studies have demonstrated that some postbiotics and paraprobiotics exhibit anti-inflammatory, immunomodulatory, anti-proliferative, antioxidant, and antimicrobial activities (Vallejo--Cordoba et al., 2020).

Paraprobiotics containing non-viable probiotic cells have potential applications in several areas, mainly in food and nutrition, e.g., as dietary supplements or feed additives. Currently, there are only a few food paraprobiotic products with health or therapeutic applications on the market (Vallejo-Cordoba et al., 2020).

For example, LAC-Shield™, a commercial heat-killed *Lactobacillus paracasei* MCC1849 (taxonomically reclassified as *Lacticaseibacillus paracasei*) has proven to be effective in improving resistance against common cold in susceptible subjects (Murata et al., 2018). The authors confirmed the safety of the continued oral intake of 1×10^{10} to 3×10^{10} cells of the product in healthy young adults. Staimune® is another patented probiotic-derived ingredient, consisting of inactivated GanedenBC30® (*Bacillus coagulans* GBI-30; taxonomically reclassified as *Heyndrickxia coagulans*). The product has been deemed safe for human consumption based on an in vitro study, which confirmed the strain did not demonstrate any mutagenic, clastogenic, or genotoxic activity (Endres et al., 2009).

Colimil® Baby, a supplement made with *Matricaria chamomilla* L., *Melissa officinalis* L. and heat-killed *Lactobacillus acidophilus* (HA122), has been demonstrated to help prevent the inconvenience of infant colic (Martinelli et al., 2017). Nyaditum resae®, a galenic preparation of heat-killed *Mycobacterium setense manresensis* was administered in several pre-clinical and double-blind placebo-controlled clinical trials to evaluate its immunogenicity in tuberculin-positive and tuberculin-negative volunteers. The findings proved that it was effective in reducing the risk of developing active tuberculosis (Tukvadze et al., 2016). Lacteol™, a paraprobiotic product prepared from heat-killed *L. acidophilus* LB cells, offers efficacy in the treatment of acute and persistent watery diarrhoea associated with several intestinal infectious diseases. Experimental in vitro and in vivo studies support that *L. acidophilus* LB displays antibacterial activity, including antibiotic-like and cell-regulating activities (Liévin-Le Moal, 2016).

3.3. Beer and wine yeasts

For thousands of years, yeasts have served as cell factories for beer and wine production (Nielsen, 2019).

As early as 1990, a lager strain genetically engineered for dextrin utilization was approved for production of low-calorie beer in the UK (Gorter de Vries et al., 2019). The following years have seen a large amount of research towards GM brewing yeasts, supported by the ever-increasing need of brewers to offer more variety in the sensory characteristics of their products. Recently, two GM brewing strains have been approved for commercial use in the US. Berkeley Yeast has developed a GM yeast producing the primary hoppy beer flavor components, linalool and geraniol, enabling a reduction in hop utilization while maintaining the aromatic character of the beer (Denby et al., 2018). Lallemand has developed a GM yeast producing lactic acid and ethanol in a single strain, eliminating the need for co-culture in the production of sour beer (Alperstein et al., 2020). The GRAS (Generally Recognized As Safe) status of commercial GM brewing strains in the US is dependent on a thorough evaluation of the yeast's development, manufacture, use, toxicity/allergenicity, absence of antibiotic resistance, inactivation, and genetic stability (Browning et al., 2022).

As Schoeman et al. reported, two GM wine yeasts were cleared and given GRAS status by the US FDA, Health Canada, and Environment Canada. One of these commercialized GM wine yeasts has the capacity to conduct malolactic fermentation, thereby reducing the risk of biogenic amine formation by certain bacteria (Schoeman et al., 2009). The other GM wine yeast secretes much less urea, which limits the production of ethyl carbamate.

The commercialization of these two GM wine yeasts in the US and Canada has made research on and development of risk assessments for GMMs a priority. The study by Schoeman and colleagues was the first attempt to establish an objective risk assessment procedure for the release of GM wine yeasts into the environment (Schoeman et al., 2009). The behaviour and spread of GM wine yeasts was monitored in saturated

[13] Trafton, A. 2022. Engineered bacteria could help protect "good" gut microbes from antibiotics. https://news.mit.edu/2022/bacteria-good-gut-microbes-antibiotics-0411 (accessed 4 December 2023).

[14] Doughman, E. 2023. Probiotic could boost necrotic enteritis control in poultry. https://www.wattagnet.com/poultry-future/new-technologies/article/15537364/probiotic-could-boost-necrotic-enteritis-control-in-poultry (accessed 4 December 2023).

sand columns, saturated sand flow cells, and conventional flow cells. A widely used commercial *S. cerevisiae* wine yeast VIN13, a transgenic VIN13 derivative (LKA1, which carries the LKA1 alpha-amylase encoding gene of *Lipomyces kononenkoae*), a soil bacterium (*Dyadobacter fermentans*), and a non-wine soil-borne yeast (*Cryptococcus laurentii*, taxonomically reclassified as *Papiliotrema laurentii*) were compared in laboratory-scale microcosm systems designed to monitor microbial mobility behaviour, survival, and attachment to surfaces. It was found that LKA1 cells survived in saturated sand columns, but showed little mobility in the porous matrix, suggesting that the cells attached with high efficiency to sand. There was no significant difference between the mobility patterns of LKA1 and VIN13. Both *S. cerevisiae* strains either had no difference in biofilm density, or the LKA1 biofilm was less dense than that of VIN13. Overall, it was concluded that the LKA1 transgenic yeast had the same reproductive success as VIN13 in these 3 microcosms and had no selective advantage over the non-transformed parental strain (Schoeman et al., 2009).

3.4. Microorganisms for bioremediation

3.4.1. Bioremediation of contaminants

Environmental pollution has been on the rise in recent decades. Amongst the pollutants that are of environmental and public health concern due to their toxicity are: heavy metals, nuclear wastes, pesticides, greenhouse gases, and hydrocarbons (Azubuike et al., 2016). Remediation of polluted sites using microbial processes (bioremediation) has proven effective and reliable due to its eco-friendly features. The two major approaches to enhance bioremediation are biostimulation and bioaugmentation (Azubuike et al., 2016). Enhancing bioremediation efficacy with controlled use of GMMs is a promising approach. This is due to the possibility of engineering a designer biocatalyst, which can effectively degrade a target pollutant by incorporating novel and efficient metabolic pathways.

Nevertheless, the potential risks for (a) horizontal gene transfer and (b) uncontrolled multiplication of the GMM in the environment limit the application of such promising approaches. To be of practical usefulness in the field, the GMM must be able to survive and multiply in such environments. Bacterial containment systems, in which any GMM escaping an environment will be killed by induction of controlled suicide systems, will help gain acceptance for GMMs in restoring polluted environments. Furthermore, genetically engineered microorganisms that degrade a target compound could improve bioremediation efficiency (Azubuike et al., 2016).

3.4.2. Biomining

Biomining, also known as bioleaching, has found particular use in the extraction of copper, gold, uranium, silver, cobalt, and rare earth elements from minerals using arsenic/sulfide-oxidizing bacteria, archaea, and occasionally eukaryotes (Drewniak and Sklodowska, 2013; Karthikeyan et al., 2015; Martínez-Bellange et al., 2022; Schippers et al., 2013). The method involves releasing naturally occurring organisms into aqueous holding areas for the ore and is typically a more environmentally friendly alternative to traditional cyanide-based processes in the mining industry, where the waste is highly toxic. An example is the Baia Mare Aurul gold mine accident on 30 January 2000, in Romania, where a dam containing cyanide mining waste burst and released 100, 000 m^3 of wastewater into the environment. Waste from a biomining process will – similar to conventional waste - be acidic and have high concentrations of metal ions but does not contain highly toxic cyanides. In addition to the less toxic waste, advantages of biomining over a conventional process are lower energy requirements, lower capital investments, and low manpower requirements, compounded by the ability to process low-grade ores.

3.5. Plant growth-promoting and biocontrol bacteria

To sustain the growing food demand of the increasing global population when arable land is becoming scarce, it is increasingly necessary for agricultural practices to move toward a more sustainable and environmentally friendly approach while at the same time increasing productivity ("sustainable intensification"). This includes both the increasing use of transgenic plants, plant growth-promoting bacteria (PGPB) and biocontrol agents as a part of mainstream agricultural practices (Glick, 2012; Ruiu, 2018). The PGPB could offer an environmentally friendly alternative to synthetic chemical fertilizers and pesticides to promote plant growth and stress tolerance (Chandran et al., 2021). In addition, microbial biopesticides include several microorganisms like bacteria, fungi, baculoviruses, and nematode-associated bacteria. They act against invertebrate pests in agro-ecosystems and offer a broad spectrum of biocontrol for crop plants (Ruiu, 2018).

Bacteria that can promote plant growth include those that are free-living, those that form specific symbiotic relationships with plants (e.g., *Rhizobia* spp. and *Frankia* spp.), bacterial endophytes that can colonize some or a portion of a plant's interior tissues, and cyanobacteria. PGPB can be used for multiple applications, such as enhancing plant growth and nutritional quality, disease suppression and conferring abiotic stress tolerance, biomass and biofuel production and bioremediation of contaminated and degraded lands (Abhilash et al., 2016; Poria et al., 2022).

Many PGPB have been isolated, and some are widely accepted as biofertilizers, biostimulants, and biocontrol agents (Ke et al., 2021). A list of companies and organizations producing PGPB can be found in (Abhilash et al., 2016). However, applying PGPB to fields has had limited success in terms of commercial adoption (Ke et al., 2021). This is likely because the new microbes are excluded by the more resilient existing microbial communities, whose composition has been shaped over time through complex multilateral interactions with the environment (Ke et al., 2021).

Microbiome engineering is increasingly recognized as a way to give host plants PGP advantages. A major limitation for crop growth and plant productivity is the bioavailability of essential nutrients, such as nitrogen and phosphorus. Nitrogen bioavailability in soils can be improved by microbial nitrogen fixation from inorganic atmospheric nitrogen into bioavailable nitrogen forms by diazotrophs. PGPB can be improved to become specialized in nitrogen fixation, increasing the bioavailable nitrogen fraction in the soil, and thus increasing crop yield. In addition, engineered PGPB could allow the growth of crops in environmentally unsuitable areas impacted by drought or salinity (Arora and Baldi, 2015; R. Gupta et al., 2023). The use of GMMs under the deliberate release Directive 2001/18/EC is strictly regulated, which severely limits the exploitation of engineered PGPB as more sustainable alternatives to agrochemicals. For commercialization of genetically engineered PGPB strains, the question under which conditions GM strains would be suitable for environmental use needs to be resolved.

Corich and coworkers reported the first open-field release of GMMs in Italy. It covered ten years of monitoring and assessing in-field GMM dynamics from strain release to disappearance as well as the impact on resident microorganisms (Corich et al., 2007).

The released GM bacteria belong to the nitrogen fixing legume endosymbiont *Rhizobium leguminosarum* bv. *viciae*, and were engineered with non-agronomically-proficient traits, in order to assess their behaviour and fate without GMM-specific positive feedback from the plant. The authors observed that the GMM strain does not affect the microbiota at the site of introduction in ways that could be related to genetic modification. Considering the nature of the released bacterial species, and the neutral outcome of the introduced genes in the chosen habitat, the absence of GMM-related impact is in line with expectations.

The paper from Wen et al. describes the identification, development, and deployment of the first microbial product optimized to enable biological nitrogen fixation (BNF) for corn (*Zea mays*) in fertilized fields,

demonstrating the successful, safe commercialization of root-associated diazotrophs and realizing the potential of BNF in replacing and reducing synthetic nitrogen fertilizers in agriculture (Wen et al., 2021). Derived from a wild-type nitrogen-fixing microbe isolated from agricultural soils, *Klebsiella variicola* 137-1036 ("Kv137-1036") retains the capacity of the parent strain to colonize corn roots while increasing nitrogen fixation activity 122-fold in nitrogen-rich environments (Wen et al., 2021).

The sector of microbial biopesticides in agroecosystems is experiencing a significant growth and many discoveries are being developed into new biopesticidal products (Ruiu, 2018). There is a long history of using natural products as the basis for creating new pesticides but there is still a relatively low percentage of naturally derived pesticides relative to the number of pharmaceuticals derived from natural sources. Microbial biopesticides have been around for 70 years, starting with *Bacillus thuringiensis*, but they are experiencing rapid growth due to innovation and development, as well as increasing restrictions on use of synthetic chemical pesticides. When integrated into crop production and pest management programs, biopesticides offer the potential for higher crop yields and quality than chemical-only programs. Added benefits include for example the reduction or elimination of chemical residues, therefore easing export, delayed development of resistance to chemicals by pests and pathogens and shorter field re-entry, biodegradability and production using agricultural raw materials versus fossil fuels, and a low risk to affect non-target organisms, including pollinators (Marrone, 2019).

Product examples for biocontrol agents of crop plants can be found via trade associations such as the International Biocontrol Manufacturers' Association[15] and company websites, e.g. biocontrol of fungal diseases by e.g. Serifel® (*Bacillus amyloliquefaciens*), against insect attack by e.g. Velifer® (*Beauveria bassiana*) or against nematodes by e.g. VoTiVo® (*Bacillus firmus*).[16]

3.6. Microbes in body cleaning, cosmetics and hygiene products

Cosmetic and hygiene products provide other possible avenues for the use of microorganisms to replace existing ingredients, provide new formulations, or promote the skin microbiome. According to the US FDA, a cosmetic is defined as "a product (excluding pure soap) intended to be applied to the human body for cleansing, beautifying, promoting attractiveness, or altering the appearance".[17] This can include microbe-derived products, pre- and postbiotics, and probiotic microorganisms. Microbe-derived products in cosmetic use include fermentation-derived ingredients such as oligosaccharides, exopolysaccharides, biosurfactants, pigments, proteins, enzymes, hyaluronic acid, and secondary metabolites (Gomes et al., 2020; Gupta et al., 2019). Prebiotics are also increasingly used in cosmetic applications to support the growth of beneficial microorganisms and inhibit pathogenic bacteria in the skin microbiome (Rademacher et al., 2022). Postbiotics in cosmetic applications, mainly derived from lactic acid bacteria (*Lactobacillus* genera) and *S. cerevisiae,* are also used for their antioxidant, anti-inflammatory, anti-proliferative and immunomodulatory properties (Duarte et al., 2022).

There is growing interest in the use of microorganisms as probiotics in cosmetic and hygiene products, including applications for acne, atopic dermatitis, female intimate care, and halitosis. For example, Lactobio has isolated strains from the environment (food, human microbiome) for use in skincare and intimate care.[18] Using microorganisms in cosmetic products can be challenging in terms of formulation for topical delivery. Furthermore, to reduce unwanted bacterial contamination, many cosmetic products contain bactericidal or bacteriostatic preservatives (Puebla-Barragan and Reid, 2021). The bacterial composition of the skin microbiome is being extensively researched, with corresponding opportunities for probiotic applications. For instance, *Cutibacterium acnes* is one of the most common bacteria in the skin microbiome, with different subtypes regarded as pathogenic or commensal. There is evidence to show that some subtypes may contribute to skin health. A strain of *C. acnes* subsp. *defendens* XYC - W1 and a growth-arrested derivative XYCM42, which were developed using a genetic switch allowing viability but preventing cell division, were investigated for use as a topical skin probiotic to improve the skin environment and appearance (Rhee et al., 2023). A further study engineered *C. acnes* to modulate cutaneous sebum production in mice for potential application as a skin-delivered therapeutic (Knödlseder et al., 2024).

3.7. Microbes in medicinal products

Certain bacterial species, and even some archaeal species, hold promise with regards to developing treatments for human diseases. The body of knowledge in this area has drastically expanded over the past two decades, and with the arrival of NGTs, additional new pathways for medicinal applications of modified bacteria have presented themselves. Therefore, a brief overview of the current status of science, a description of the basic principle of the use of GMMs in medicine and a discussion of the potential impact on patient and environmental safety is provided below.

3.7.1. Basic principle of the use of GMMs in medicine

GMMs can be used as vehicles to deliver therapeutic genes or specific compounds to patients to elicit a therapeutic effect. Their use has been effective or is being tested in various therapeutic areas including oncology, autoimmune, inflammatory, and infectious diseases, as well as vaccine delivery and within the practice of preventive medicine (Cook et al., 2018; Dieye et al., 2022; Duong et al., 2019; Howell and Forbes, 2022; Kalia et al., 2022; Moghimipour et al., 2021; Plavec and Berlec, 2019; Wu et al., 2022).

Several bacterial species and associated strains have turned out as promising therapeutic vehicles. Among the range of candidates, the most prominent examples include *Clostridium* sp., *Salmonella* sp., *Listeria* sp., and *Lactococcus* sp.. All these bacteria have fully sequenced genomes and are in general very well characterized. For potentially pathogenic strains, i.e., *Salmonella* species, attenuated versions that do not pose a threat to the exposed organism have been developed and used in laboratories worldwide for several decades (Cook et al., 2018; Dragunsky et al., 1990; Hajra et al., 2021; Howell and Forbes, 2022; Kalia et al., 2022; Kaper et al., 1994; Moghimipour et al., 2021).

3.7.2. Examples: Salmonella and Listeria applications in cancer treatment

Salmonella enterica and *Listeria monocytogenes* both exhibit the useful trait of enhanced survival in low-oxygen environments, as is the case within solid tumors. *Salmonella* grows in solid tumors at a ratio of 10 000 to 1 when compared to their growth in healthy tissues (Forbes et al., 2003). This characteristic can be exploited by modifying the genome of these bacteria to carry genes with therapeutic effect into the tumor. Both species have been orally and systemically administered in clinical trials (Meng, 2005; Shams et al., 2001; Stavru et al., 2011). The genome can be modified to deliver cytokines and/or tumor-associated antigens into

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[16] BASF, 2023. BioSolutions by BASF. https://agriculture.basf.com/global/en/business-areas/crop-protection-and-seeds/BioSolutions.html (accessed 4 December 2023).

[17] FDA, 2021. Importing Cosmetics. https://www.fda.gov/industry/importing-fda-regulated-products/importing-cosmetics#cosmetic (accessed 4 December 2023).

[18] Lactobio, Live probiotic bacterial strains. https://www.lactobio.com/pages/products#live-probiotic-bacterial-strains (accessed 6 December 2023).

the tumor to direct the patient's immune system to the normally immune-evasive cancer tissues (al-Ramadi et al., 2009; Fensterle et al., 2008; Loeffler et al., 2007, 2008, 2009; Niethammer et al., 2002; Nishikawa, 2006; Seavey et al., 2009). There are also approaches under development that make use of delivering small silencing RNAs to shut down oncogenic expression or to deliver toxins into the tumor to eliminate the cancer (Blache et al., 2012; Ebelt et al., 2020; Phan et al., 2020).

In addition to using attenuated strains, these bacteria can be equipped with genetic safeguards to minimize the risk to patients and the environment (see also section 1.4 above). For example, it is possible to activate and shut down expression of specific genes by transcriptional control via external signals. Simple switches and more complicated genetic circuits can be implemented for therapeutic genes in order to deliver the effect (i.e., specific gene expression) at a specific timepoint or under specified conditions. Such switches can also be implemented to control housekeeping genes to ensure the bacteria can be killed by removing the external signal (Gurbatri et al., 2022). Genetic modifications like the latter automatically render these organisms incompetent for survival outside of the patient. The risk of unhindered spread in the environment is thereby virtually eliminated.

Essentially the same principles of genetic modification can be used for the treatment of other diseases, e.g., autoimmune diseases of the intestines (Cook et al., 2018). In these cases, it makes sense to use different bacteria like *Lactococcus* sp., which have the ability to survive under the conditions encountered in the human gut and which can be modified to deliver the desired therapeutic effect. Other applications include the use of various types of attenuated GM bacteria as vaccines. Vaxchora, for example, a vaccine approved in the EU, contains a maximum of 2×10^9 CFU of genetically modified *Vibrio cholerae*. An environmental risk assessment was performed according to the relevant guidelines (see below) and showed that the bacterial concentration was substantially reduced after three days. Any release of the bacteria into the environment was therefore considered as negligible.

3.7.3. Patient and environmental safety

The rigorous non-clinical and clinical testing and subsequent regulatory review process are designed to demonstrate and ensure that only safe and effective medicines are granted marketing authorization for use in both human and animal patients. Similarly, a rigorous review process applies to the assessment of the potential environmental risk posed by this type of GMO as per the EU Directive 2001/18/EC.

Genetically modified bacteria, such as those used in medical applications that may enter the environment as a result of imprudent handling, patient shedding or other inadvertent release are not expected to be capable of surviving in the natural environment given their limited genetic fitness, caused by the reduction and modification of the genetic content of their genomes and the addition of therapeutic genes and potential genetic switches. A list of environmental release approvals issued from EU national agencies for various GM bacterial applications is provided in Table 1.

Attenuated strains of the relevant bacterial species have been in use in laboratories worldwide for several decades with no discernible negative impact on the human population or the natural environment. The use of complex genetic circuits to control every relevant aspect of the GM bacterium, including elaborate kill-switches, will most likely grow in the future, enabling the current negligible risk level to be maintained. NGTs such as CRISPR-based systems make the modification of bacterial genomes, particularly marker-less modifications and the implementation of more complex genetic circuitry, easier and faster than ever before.

The clinical impact of GM bacteria is mixed, and the number of clinical applications tested to date remains low. Overviews of ongoing and completed clinical trials with GM bacteria can be found in several publications (Duong et al., 2019; Howell and Forbes, 2022; Wu et al., 2022). Nevertheless, the impact of NGTs should not be underestimated as it might give a boost to this area of research.

Table 1
Non-exhaustive list of deliberate release approvals granted by EU member states for GM bacteria used in medical applications, including vaccines.

Bacterial Species	Notification numbers of approved deliberate release in the EU	Date of consent given
Actinobacillus pleuropneumoniae	B/ES/13/21, B/ES/07/46	2014
Actinobacillus pleuropneumoniae	B/BE/11/V3, B/ES/08/48	2012
Bacillus subtilis	B/DE/16/PEI2797	2016
Salmonella enterica	B/GB/15/R47/01/NI	2015
Lactococcus lactis	B/BE/07/BVW1	2007
Listeria monocytogenes	B/ES/17/20	2018
Listeria monocytogenes	B/RO/17/02, B/ES/17/02	2017
Rhodococcus equi	B/DE/10/213	2012
Salmonella enterica	B/GB/20/48/01	2020
Salmonella typhi	B/GB/03/R35/02	2003
Salmonella enterica	B/NL/19/003, B/NL/19/007, B/NL/18/004,	2019
Salmonella enterica	B/DE/16/PEI2516	2016
Salmonella enterica	B/DE/15/PEI2509	2016
Salmonella enterica	B/DE/11/PEI1393	2013
Staphylococcus aureus	B/NL/03/02	2004
Streptococcus pneumoniae	B/GB/18/R51/01	2018
Vibrio cholerae	Marketing approval EMEA/H/C/003876	2020

4. Recommendations for regulatory oversight

Most applications of viable microorganisms in the EU, particularly in the food and feed sector, currently rely on wild-type strains or classically mutagenized strains considered as GMMs, but excluded from the scope of Deliberate Release Directive 2001/18/EC. Although Directive 2001/18/EC is in place and applies to products containing viable GMMs, no procedures and guidelines are available for submitting applications for such products. In particular, appropriate tools for the environmental risk assessment are missing. This represents a major barrier for use of state-of-the-art biotechnology techniques, inhibiting innovation and R&D, and resulting in reduced opportunities in the EU for products such as probiotics and food cultures, all of which would contribute significantly to support the ambitions of the EU Green Deal.

The current EU regulatory framework for biotechnology products (under the Deliberate Release Directive 2001/18/EC and the Contained Use Directive 2009/41/EC) is outdated, as evidenced most clearly by the emergence of NGTs, which sometimes question the actual distinction between GMOs and non-GMOs. The terms GMOs and non-GMOs are no longer fit for purpose. Even more clearly than in plants, the boundaries between "genetically modified" and "conventional" microorganisms have become blurred. The current framework is primarily process-based as opposed to product-based. Future GM legislation should be developed by putting the characteristics of the organisms in focus and providing a specific legal framework for microorganisms, thereby preventing the stigmatization of "GM" products by avoiding the terms of GMO and non-GMO and by providing regulatory clarity. Furthermore, the regulatory requirements and burden should be technologically and economically feasible and proportionate to the risk.

Nevertheless, the current safety assessment framework in the EU provides all the necessary assurances for environmental protection, occupational health and safety, the safety of local residents in agricultural areas, and the main aspects of food and feed safety (Scheepmaker et al., 2016).

The general key principles for an ERA can be used for food and feed related applications:

i. Identification of GMM characteristics which may cause adverse effects;

ii. Evaluation of the potential consequences of each adverse effect;
iii. Evaluation of the likelihood of the occurrence of each identified potential adverse effect;
iv. Estimation of the risk posed by each identified characteristic of the GMM(s);
v. Application of management strategies for risks arising from the deliberate release/marketing of the GMM(s); and
vi. Determination of the overall risk of the GMM(s).

As summarized in this paper, a wealth of scientific information and experimental data is available to allow the risk assessment and risk management of GMMs for deliberate-release applications.

Based on the current scientific knowledge, it can be concluded that most engineered microbes do not need to be regulated more strictly than the microbial strains already on the market. Exceptions would be strains derived from microbial species that are pathogenic to humans and animals and/or which include acquired genes of concern. Microbial strains intended for use in food or feed applications should not contribute further to the reservoir of antibiotic resistance genes already present in the gut flora of humans and animals as well as in the broader environment.

In addition, the ERA must cover the possibility that the GMM survives and persists in the environment, and eventually multiplies and/or mates with indigenous microorganisms. It must also take into consideration products for which medium-term persistence in the environment is needed to afford the beneficial effect (e.g., biologicals, bio-fertilizers or probiotics).

Finally, methods exist for effective GMM traceability and control. However, there is a need for further development and validation to enable specific and reliable detection, identification, and quantification for each GMM individually. The efficiency and efficacy of these methods must be kept in mind, considering limited control capacities and hundreds of potential upcoming products. New biocontainment strategies may offer additional safe and effective strategies for environmental use of GMMs.

5. Conclusion

There are many current and future market applications for GMMs, whether in terms of fermentation products, or in terms of the microorganisms themselves being the product. This review covers current knowledge regarding microorganisms in the environment, including possible routes of release, environmental risk assessment, availability of specific detection methods, and in case needed, potential biocontainment strategies. It also provides an overview of existing and possible future deliberate release applications across sectors to examine the safety knowledge that can be gained from environmental risk assessments. It concludes that there is sufficient scientific knowledge and safety data available on this topic to support an examination of the regulatory approach for GMMs in Europe. In general, and excluding pathogenic organisms, there is no scientific justification for a stricter regulation of GMMs compared to their non-GM counterparts, particularly since the distinction between GMO and non-GMO is blurred by the continual evolution of genetic engineering techniques. Current regulations are based upon scientific evidence regarding the safety of the product but focus too much on technologies used for product development. A more product-centric approach is urgently required to help the EU achieve its innovation and sustainability goals. The current EU regulatory approach prevents many of these applications from reaching the market, even though many of the applications outlined in this review can contribute to European sustainability ambitions outlined as part of the EU Green Deal, and in some cases have already reached the market in other jurisdictions. Therefore, building on the scientific knowledge presented in this review to work towards a supportive and science-based regulatory framework for deliberate release of GMMs would support EU innovation and sustainability goals.

CRediT authorship contribution statement

Alexandra Lensch: Conceptualization, Writing – original draft, Writing – review & editing, Project administration. **Hanna Abbas Lindfors:** Writing – original draft, Writing – review & editing. **Elke Duwenig:** Writing – original draft, Writing – review & editing. **Tobias Fleischmann:** Writing – original draft, Writing – review & editing. **Carsten Hjort:** Writing – original draft, Writing – review & editing. **Sirpa O. Kärenlampi:** Writing – original draft, Writing – review & editing. **Lucie McMurtry:** Conceptualization, Writing – original draft, Writing – review & editing, Project administration. **Emily-Denise Melton:** Writing – original draft, Writing – review & editing. **Mikael Rørdam Andersen:** Writing – original draft, Writing – review & editing. **Ryan Skinner:** Writing – original draft, Writing – review & editing. **Markus Wyss:** Writing – review & editing. **Richard van Kranenburg:** Writing – original draft, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The views and opinions expressed in this document are those of the authors in their personal capacity. None of the observations, opinions or conclusions expressed herein are to be attributed to or construed as the views or positions of any organization, association or company. AL, HAL, ED, CH, EDM, MRA, RS, MW, RVK are employed by companies which commercially produce microorganisms and products of interest by industrial fermentation. LM is employed by an international trade association representing the biotechnology industry in Europe.

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