Effects of European eel egg and larval stocking density on rearing water, offspring bacteriome and derived immune response

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Feature Review

Horizon scanning the application of probiotics for wildlife

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The provision of probiotics benefits the health of a wide range of organisms, from humans to animals and plants. Probiotics can enhance stress resilience of endangered organisms, many of which are critically threatened by anthropogenic impacts. The use of so-called 'probiotics for wildlife' is a nascent application, and the field needs to reflect on standards for its development, testing, validation, risk assessment, and deployment. Here, we identify the main challenges of this emerging intervention and provide a roadmap to validate the effectiveness of wildlife probiotics. We cover the essential use of inert negative controls in trials and the investigation of the probiotic mechanisms of action. We also suggest alternative microbial therapies that could be tested in parallel with the probiotic application. Our recommendations align approaches used for humans, aquaculture, and plants to the emerging concept and use of probiotics for wildlife.

Highlights

Probiotics can enhance the resilience of endangered wildlife.

The use of so-called 'probiotics for wildlife' is a nascent application.

Incorporating reliable negative controls in the experimental design is essential to validate probiotic effects.

Other challenges include culturing and selecting probiotics, risk assessment, optimization and scaling, and understanding the mechanisms of action.

Additional microbial therapies (e.g., postbiotics) should be developed and tested as alternative treatments to protect wildlife.

Probiotic interventions

Most organisms rely on their resident microbiome, giving rise to the 'metaorganism' or 'holobiont' [1–4]. Microbes contribute to host health and development by several means, including provisioning of nutrients, promoting development and growth, detoxifying, and mitigating disease [5]. For example, specific rhizosphere microbiota can increase drought tolerance in plants, bee microbiota can influence host immunity, and the human gut microbiome can protect against disease [6–10]. In addition, microbiomes are both resilient, flexible, and quick to respond to environmental changes [11–13], which, together with their large metabolic potential, constitute the main premise of the effective use of **probiotics** (see Glossary) [14] and other microbial therapies to modulate host functioning [7,8,10,15–18].

Probiotics are defined as live microorganisms that can confer a health benefit to the host [19]. The probiotic concept is founded on a central pillar of two key microbiome-based modulation strategies: (i) restore the 'native' microbiota following a disruption (e.g., infection or antibiotic treatment), and/or (ii) enhance host resistance to external stress (e.g., increase disease tolerance) [20–26]. Active manipulation of microbes is achieved in a number of ways, including: (i) altering environmental conditions [27,28], (ii) applying abiotic agents that select for or against specific microbial activity, such as **prebiotics** (i.e., substrates that can select beneficial microorganisms to the host) [19] and **postbiotics** (i.e., dead cells or their components that trigger benefits to the host) [29,30], (iii) transplanting healthy or beneficial microbiomes [31–33], or (iv) inoculating host organisms with probiotics (i.e., a single strain or cocktail of living microbes) [19,34]. Probiotics typically comprise isolated and cultured mutualistic microbes of the target organisms [15,20,35].

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However, they can be sourced from other hosts, sites, and surrounding environments [20] or selected based on specific microbial traits or mechanisms that putatively benefit the host [35,36]. To date, the most commonly used probiotic organisms are bacteria, but they can be sourced from any microbial domain [e.g., archaea, microalgae, protists (single cell eukaryotes), or fungi] [37,38] and assembled in a customized way [39].

Provision of probiotics is not a new concept, and it has been broadly applied to improve host health in human health care [7,21,40,41], agriculture [6,42,43], and aquaculture [44–47] for decades. More recently, probiotics have been considered for endangered wildlife organisms [8,16,23,35,48,49]. The development of novel interventions to protect at-risk wildlife currently covers bees, amphibians, bats, plants, and corals [8,16,50–52], and is particularly urgent considering their ecological relevance, the current status of the terrestrial and marine habitats they inhabit, and the global loss of biodiversity at large [8,14,16,50–54]. Probiotics can play a role in conserving and restoring populations [49,50] by bridging the time needed to reduce ecosystem-scale pressures (e.g., climate change, disease outbreaks, and pollution) [49,50].

Reef-building corals, for example, and the ecosystems they build, support a vast biodiversity of marine life (>30% of all marine eukaryotic species) [52,55,56] and have been declared by the Intergovernmental Panel on Climate Change (IPCC) to be at the highest risk of decline due to climate change, compared with other marine ecosystems [57]. The window of opportunity to act to maintain coral reefs, as we know them, is rapidly closing [56], and they could significantly benefit from the rapid development of probiotics that aim to increase their stress resilience. On land, the growing loss of wild and managed pollinating insects drives declines in biodiversity and critically jeopardizes food security on our planet [58]. Insect populations are decimated by the consequences of climate change, but also by human-made chemical pollutants and the spread of disease agents, and would also benefit from probiotics that can boost their immunity and stress resilience [59,60]. Similarly, other wildlife, such as several amphibian species, are critically threatened, in particular by deadly and widespread diseases exacerbated by human activity, climate change, and pollution [54,61–64], which may eventually lead to the extinction of populations or entire species [65].

The use of probiotics for endangered wildlife is a nascent field of research. Even basic aspects of their study, such as the use of standards for its development, testing, risk assessment, and deployment have not yet been fully defined [49]. The first step towards such a standardization has been initiated specifically for amphibian disease mitigation [66]. To further streamline and accelerate the development of these emerging applications across taxa, we review the most recent interventions for several wildlife hosts (mainly corals, amphibians, bees, and bats) to identify the state-of-the-art, specific challenges in administering probiotics for wildlife, and potential pitfalls of experimental design. We then provide recommendations for a comprehensive experimental assessment of this emerging research field and make suggestions that include investigation of the mechanisms of action and alternative microbial-based strategies (see Outstanding questions).

Emerging probiotic applications in wildlife

The first probiotic applications in wildlife have been implemented to treat infectious diseases – for example, the deadly white-nose syndrome (WNS), caused by the fungus *Pseudogymnoascus destructans*, which threatens the survival of entire populations of bats in North America [61,67,68]. The application of probiotics in this case has significantly reduced the severity of WNS and increased the survival rate of brown bats (*Myotis lucifugus*) in both laboratory tests [69] and field trials [16], making the use of probiotics one of the most promising solutions to

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treat WNS. Further, probiotics have been successfully used in laboratory and field trials for honey bees (*Apis mellifera)* infected with pathogens (e.g., *Nosema ceranae*, *Serratia marcescens*, or *Paenibacillus larvae*). In all cases, the use of probiotics increased the survivorship of infected bees [8,70–73]. Additionally, probiotics also protected bees from pesticides by improving their immune response and detoxification system, increasing the number of survivors, and extending their lifespans [74]. In amphibians, antagonistic microbes or coculture of symbiotic-associated bacteria successfully inhibited the deadly cutaneous fungal pathogen *Batrachochytrium dendrobatidis* [75] and increased the survivorship of boreal toads (*Anaxyrus boreas*) following infection with the same *B. dendrobatidis* pathogen [76]. However, other studies targeting the same disease in Panamanian golden frogs (*Atelopus zeteki*) [51] and yellow-leg frogs (*Rana sierrae*) [77] did not detect positive effects of the probiotic application. This implies that probiotic efficiency may vary according to the target host species, their resident microbiomes, probiotic selection, and/or type of application. Despite the observed variation in success, bioaugmentation of beneficial bacteria for frogs in their surrounding environment (soil) was shown to decrease the presence of the pathogen, suggesting the potential of inoculating probiotics in the environment as a preventative measure against infections [78]. Similarly, a zoosporic (fungal) disease that has caused havoc in European limnic ecosystems over the past century could also be potentially treated using probiotics. The invasive fungal pathogen *Aphanomyces astaci* has decimated wild stocks of the European noble crayfish *Astacus astacus*, a keystone species and ecosystem engineer that is also an economically significant species [79]. In this particular case, the discovery of inhibitory bacteria from the crayfish carapace not only provides a positive outlook for aquaculture disease management but it may emerge as a probiotic strategy to help save Europe's wild crayfish population [80].

Probiotic applications in wildlife can also improve the productivity and performance of host organisms rather than targeting a specific infection. To date, such enhancing approaches have mostly focused on plant species. For example, the addition of mycorrhizal fungi and/or endophytic bacteria can double root growth while reducing water requirements of *Retama sphaerocarpa*, a drought-adapted legume [81] and also increased the growth of clover (*Trifolium* spp.) twofold to fivefold in heavy-metal-polluted soil [82–84]. The isolation of microbiota from plants living in extreme environments is another strategy that is actively explored to select probiotic or functional candidates with desired functions [85,86]. For instance, bacterial isolates from various desert plants increased salt stress tolerance when applied to the model plant species *Arabidopsis thaliana*, illustrating the possible use of wildlife-sourced probiotics to increase agricultural production and food security [87]. Another example showed that bacteria isolated from stress-tolerant organisms colonizing extreme habitats such as lichens can be used to protect crops against abiotic stress [88], and have been successfully commercialized as stress-protecting agents (SPAs) [89]. This strategy can also be applied to tree species, helping these important ecosystem engineers to withstand the stresses of cold, drought, and heat in their natural habitat. This has been currently applied in the form of a soil microbiome transplantation [90].

Probiotic applications aiming to improve the performance of strictly aquatic or marine organisms present another challenge with respect to delivery and dilution effects in the aquatic habitat. To date, most successful case studies of probiotic applications have been conducted in farmed aquatic animals. For example, gilthead sea bream (*Sparus aurata*) larvae and fry reared with probiotic addition by live food (rotifer and *Artemia)* showed increased survival and growth rates [91] and stress tolerance [92]. Similarly, farmed bullfrog tadpoles (*Lithobates catesbeianus*) fed a diet supplemented with selected autochthonous lactic acid bacteria responded with improved hematological parameters, increased length and density of intestinal microvilli, and overall higher weight gain [93]. Further, the addition of probiotic bacteria to fish feed can increase their fecundity while

Glossary

Alternative microbial-based therapies: strategies that aim to manipulate the microbiome communities of an organism with the intention of improving health, increasing performance, preventing infection, or re-establishing the homeostasis between the host and its microbiome. Approaches include the use of prebiotics, selective antibiotics, bacteriophages, small-molecule inhibitors, or microbiome transplants, for example, [163] (also known as 'microbiome therapeutics' or 'microbiome engineering').
'No addition' control group: control treatment that includes a group of subjects that receive no treatment or intervention.
Placebo: the definition of 'placebo' in human trials can include a non-drug/treatment and includes psychological aspects associated with the use of a certain probiotic [162]. It can be extrapolated to mimicking the delivery procedure, which also includes the use of the same delivery carrier.
Postbiotics: treatments involving inanimate microorganisms and/or their components to confer a health benefit to the host [30]. These treatments are normally prepared by autoclaving, sonicating, or heat-inactivating microbial cells.
Prebiotic: a substrate that is selectively utilized by host microorganisms conferring a health benefit [115].
Probiotics: live microorganisms that, when administered in adequate amounts, confer a health benefit to the host [19].

changing the gene expression of neuropeptide hormones and metabolic signals, as demonstrated in the zebrafish (*Danio rerio*) model system [94]. Another example is the application of probiotics to reef-building corals, which was initially established in the laboratory to mitigate the effects of oil pollution [23]. More recently, the concept of microbiome restoration and rehabilitation for corals [20,23,24,26,95–98] was followed by a specific framework [95] describing potential beneficial microbial traits mainly aiming to enhance coral thermal resistance. A promising previous example, where thermal resistance of a host was enhanced by the replacement of bacterial symbionts, was provided for the pea aphid, an insect model organism [99,100]. The protective and enhancing effect of several putative bacterial probiotics for corals was validated [20], which was later expanded to a wide range of probiotic consortia beyond bacteria, including dinoflagellates, filamentous fungi, and yeast [15,20,23,38,97,101,102]. Overall, corals treated with probiotics experience higher growth rates, lower mortality following thermal stress, and overall lower stress responses when exposed to combined stressors of heat and pathogen loads or after exposure to a simulated oil spill [15,20,23,38,101–103].

The majority of these probiotic applications are still in developmental and undergoing laboratory testing. Streamlining and standardization of study approaches across taxa promises to accelerate these developments and bring them closer to real-world or large-scale application and, hence, is one of the key foci of this review.

Challenges in developing and applying probiotics for wildlife

In many wildlife probiotic studies, the effects of the probiotic inocula can be confounded by other factors due to the complexity of the host lifestyle and environment. In these cases, certain experimental designs can be insufficient to control for potential nontarget and confounding effects, leaving questions regarding administration, dosing, and efficacy unanswered. Despite the promising results of novel probiotic applications, many challenges still need to be addressed. Currently, approaches, validation, and risk assessment strategies for emerging probiotic applications for wildlife are not fully standardized. Discrepancies in study designs are particularly reflected in the use of negative controls. The use of a **placebo** control has been common in insect, coral, and amphibian studies [15,20,38,70–72,76,97,101,102]. However, **'no addition' control groups** and a combination of 'no addition' plus placebo have also been applied [8,23]. In the following, we highlight why the validation should focus on applying inert negative controls, that is, a placebo. Further, we advocate that the underlying mechanisms, colonization aspects, nontarget effects, application strategies, and alternative microbial therapies should be explored in order to advance the field.

Culturing, selecting, and assembling probiotics for wildlife

Isolation, cultivation, and the careful selection of promising probiotic candidates for application are the first steps in any probiotic development protocol. Increasing culturability can certainly increase the range of isolates that can be considered as probiotics [104]. For this reason, the focus is typically on designing and utilizing specific modified culture media and applying more efficient culturing tools to increase the recovery of potential probiotic candidates [104,105]. The screening of probiotic candidates for specific traits is time-consuming and requires previous knowledge of the causative agent or mechanism disrupting the health of the host (pathogen, pollutant, or other metabolic stresses), which is not always known.

The use of microbial consortia is often desirable or recommended, as this may combine different (and complementary) beneficial traits [106], increasing the chances that at least one of the selected strains will promote recipient health [95]. The ideal approach is based on the bioaugmentation of the native (beneficial) consortia by aiming to increase the probability that these members

of the microbiome are retained under environmental changes and/or the presence of pathogens. When designing probiotic consortia, growth-inhibition tests are recommended to ensure compatibility among the isolates. Moreover, it is important to highlight that, although some exogenous probiotics may be eventually used, the use of native, commonly found bacteria, that have never been associated with disease in any living organism, is highly recommended [14,49].

Identifying keystone species in the microbiome, potentially symbiotic or responsible for health and resilience, is another promising way to select probiotics. Native members of the host 'beneficiome' (i.e., microbes associated with healthy hosts) [26] come with the promise of sustainable success as they tend to be more easily enriched in the recipients' microbiomes [26,35,98,107]. For instance, native bacteria of the mammalian gut and amphibian skin can be safe and effective. Bovine tuberculosis can be reduced in wildlife and livestock using strains of *Pediococcus* and *Lactobacillus*, isolated from European badgers, a natural reservoir of the disease agent, thanks to their antimicrobial and immunoregulatory activities [e.g., modulation of pro-inflammatory markers NF-κB and interferon (IFN)] [107,108]; and native symbiotic amphibian skin microbes can effectively mitigate fungal diseases [66]. Similarly, the bacterial seed endophyte, *Sphingomonas melonis*, a native microbe in rice plants that is transmitted across generations, confers pathogen resistance via the supply of anthranilic acid that interferes with gene-regulatory mechanisms in the seed-borne pathogen *Burkholderia plantarii* and has been proposed to protect crops from global disease threats [18]. With regard to the selection of desired probiotic traits, the reef-building coral perspective has offered a comprehensive overview, including universal traits that could possibly benefit other hosts, too: microorganisms that are able to produce antioxidant molecules (e.g., catalases, superoxide dismutases) or synthesize compatible solutes (e.g., betaines, floridoside, dimethylsulfoniopropionate) promise to help increase tolerance of reactive oxygen species (ROS); microbial production of mycosporine-like amino acids and carotenoids can offer UV and photoprotection of hosts; and quorum quenching or bacterivory can disrupt proliferation of opportunists and pathogens [24,95,109].

Validation of probiotic effects

Experimental design planning for probiotic studies is not straightforward, as microbiomes are highly responsive and can be altered by small changes in environmental conditions, or due to the addition of substrates or nutrients, which are often contained in the growth media of the probiotics, or other compounds contained in carrier solutions. Another problem is that other confounding factors can potentially lead to nonspecific microbiome shifts and have consequences for the holobiont, such as physiological responses of the hosts (e.g., growth rate, transcriptomic or metabolomic shifts, photosynthetic capacity/yield), which will differ with the host genotype. These caveats call for well-replicated and controlled study designs to differentiate between probiotic-specific and confounding effects. Thus, the success of probiotics can be accurately determined only if the efficacy in the treatment group can be disentangled from the effects of other factors, especially those universal to most experiments, such as: (i) the physical treatment procedure on its own, (ii) the delivery/carrier medium of the probiotic, (iii) the environment where the treatments are performed, and (iv) the intrinsic variation of the individual subjects which are part of the experiment. To minimize or eliminate the effects of any confounding variables [110], negative controls should be as inert as possible (Figure 1). Placebo control groups were the most commonly utilized negative control across different wildlife studies [15,72,77], followed by 'no addition' control group [23,73] (Table 1). A placebo treatment is an inert control that accounts for the confounding factors that are universal for most experiments, while 'no addition' controls include a group of subjects that receive no treatment at all. This means that such studies relying on 'no addition' controls cannot rule out the effect of the administration process (handling and environment) and/or the carrier solution (if relevant).

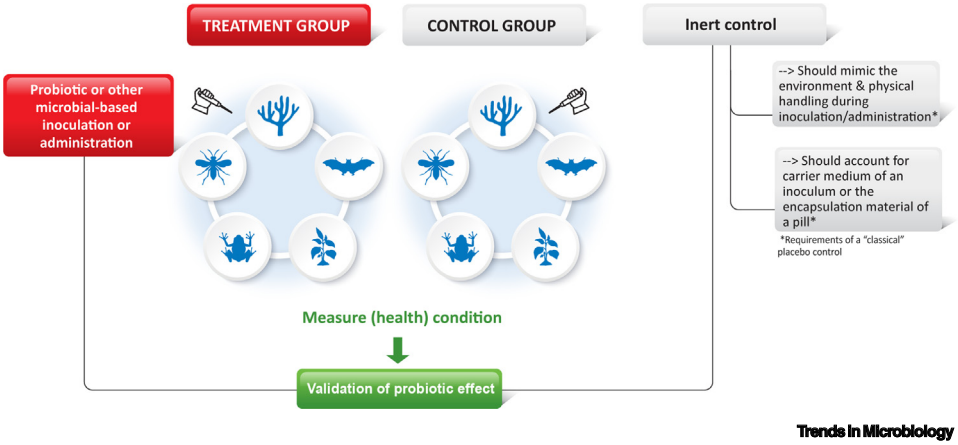

Figure 1. The importance of using inert negative controls to validate the effect of probiotics (or any other microbial therapy). Negative controls should not introduce any confounding factor. Dead microbial cells (i.e., postbiotics) or bacterial fractions (e.g., supernatants) contain bacterial cellular components that trigger specific biological responses. Live and dead microbial cells, as well as fractions of microbial cells, of the same probiotic preparation can promote beneficial traits through different mechanisms and are therefore different treatments, not inert negative controls. The validation of a probiotic or other microbial therapy can be achieved when the treatment provides improvements in the measurable phenotypic/health responses of the holobiont when compared with an inert control.

In a few probiotic studies, inactivated probiotic cells have been utilized as the only 'control' treatment [81,84]. This differs from an inert control/placebo, since dead microbial cells release bacterial cellular components that have been consistently reported as triggers of specific biological responses. In some cases, these compounds have been known to show similar, or even stronger, effects than those promoted by living cells [111–113]. Also, the deactivation method of cells is significant as this leads to the release of different types of cell components. For example, when Gram-negative cells are lysed, they release components of their outer membrane, which contains the endotoxin lipid A. This endotoxin is incredibly potent and will typically elicit a strong immunological response, even if it is derived from a nonpathogenic bacterium. However, lipid A is only released from lysed cells and represents one of the significant differences between live and 'inactivated' cells [114]. These effects are commonly referred to as 'postbiotic' effects.

Postbiotic effects and their underlying mechanisms of action

By definition, probiotics are live microorganisms; conversely, postbiotics are inactivated cells or microbial components that confer a health benefit to the respective host [30,115]. Responses promoted by postbiotics have been widely reported in human and plant studies [111,115–125] in which immunological and other bioactive effects of bacterial metabolites [111] or beneficial shifts in the native microbiome [124] were the main mechanisms underlying health improvements. Postbiotic bacterial components identified as triggers of biological responses and microbiome shifts include lipopolysaccharides, lipoteichoic acids, peptidoglycans, and exopolysaccharides [111,126–128]. Since the use of postbiotics does not fulfill the criterion for a negative control (which implies a 'blank' treatment), they should be considered as another microbial therapy and, exactly as probiotics, be compared with a placebo. Testing such alternative microbial treatments can shed light on whether nonviable microorganisms or microbial cell extracts can also elicit a beneficial effect. Indeed, it has been demonstrated that both live and dead cells of the same probiotic preparation can promote beneficial traits, although this is often through different mechanisms. For example, live cells can restructure the human gut microbiome and exert a host immune response, whereas dead cells can trigger an anti-inflammatory response [117]. In fish aquaculture, the administration of dead cells (which resulted in a beneficial effect) also measurably stimulated immunity; for

Table 1. Summary of probiotic studies in wildlife, including details about the type of probiotic species administered, control treatments, and main effects on the host reported[a]

Host	Aim of probiotic application (in case of diseases, details on pathogen are given in parenthesis)	Type of probiotic (details on the use of single species or consortia are provided in parenthesis)	Probiotic species	Probiotic application	Type of control	Effect of probiotic on measured host variables: (+) positive effect (=) no difference between treatments (–) negative effect	Microbiome changes	Refs
Mammal, bat (*Myotis lucifugus*)	Treatment of white-nose syndrome (*Pseudogymnoascus destructans*)	Bacteria (single)	*Pseudomonas fluorescens*	Topical application	Placebo	(+) Increased survival (+) Reduced disease severity	NA	[69]
Mammal, bat (*M. lucifugus*)	Treatment of white-nose syndrome (*P. destructans*)	Bacteria (single)	*P. fluorescens*	Topical application	No addition	(+) Increased survival	NA	[16]
Amphibian, salamander (*Plethodon cinereus*)	Treatment of chytridiomycosis (*Batrachochytrium dendrobatidis*)	Bacteria (single)	*Janthinobacterium lividum*	Administration in surrounding environment	Placebo	(+) Reduced pathogen loads	NA	[78]
Amphibian, panamanian golden frog (*Atelopus zeteki*)	Treatment of chytridiomycosis (*B. dendrobatidis*)	Bacteria (single)	*Chryseobacterium* sp., two *Pseudomonas* spp. and *Stenotrophomonas* sp.	Topical application	Placebo	(=) Survival (=) Pathogen loads	No	[51]
Amphibian, boreal toad (*Anaxyrus boreas*)	Treatment of chytridiomycosis (*B. dendrobatidis*)	Bacteria (single)	*J. lividum*	Administration in surrounding environment	Placebo	(+) Increased survival	NA	[76]
Amphibian, yellow-leg frog (*Rana sierrae*)	Treatment of chytridiomycosis (*B. dendrobatidis*)	Bacteria (consortium)	*P. fluorescens, Pedobacter cryoconitis, Chryseobacterium* sp., *Iodobacter* sp.	Topical application	No addition	(=) Survival (=) Pathogen loads (+) Immune modulation through skin defense peptide	No	[77]
Insect, honey bee (*Apis mellifera*)	Treatment of American foulbrood disease (*Paenibacillus larvae*)	Bacteria (consortium)	*Lactobacillus plantarum, Lactobacillus rhamnosus*, and *Lactobacillus kunkeei*	Ingestion	No addition and placebo	(+) Increased survival (+) Reduced pathogen loads (+) Upregulation of immunity	Yes	[8]
Insect, honey bee (*A. mellifera*)	Treatment of nosemosis (*Nosema ceranae*) and intoxication (insecticide and fungicide)	Bacteria and yeast (single)	*Saccharomyces cerevisiae, Saccharomyces boulardii, L. plantarum, Bacillus pumilus*, and *Pediococcus acidilactici*	Ingestion	Placebo	(+) Increased survival (+) Reduced pathogen loads (+) Upregulation of immunity and detoxification genes	No	[74]
Insect, honey bee (*A. mellifera*)	Treatment of nosemosis (*Nosema* spp.) in field and improvement of physiological parameters in lab trials	Commercial probiotic	Not specified (EM® probiotic for bees)	Ingestion and administration in surrounding environment	Placebo	(+) Reduced pathogen loads in the field (+) Improved physiological parameters in lab (–) Increased mortality in the lab	NA	[70]

Insect, honey bee (*A. mellifera*)	Treatment of nosemosis (*N. ceranae*)	Bacteria (commercial probiotics, single and consortium)	Vetafarm® and Protexin® single-strain (*Enterococcus faecium*) and multistrain (*Lactobacillus acidophilus, L. plantarum, L. rhamnosus, Lactobacillus delbrueckii, Bifidobacterium bifidum, Streptococcus salivarius*, and *E. faecium*)	Ingestion	Placebo	(+) Increased survival (+) Reduced pathogen loads	NA	[72]
Insect, honey bee (*A. mellifera*)	Treatment of nosemosis (*N. ceranae*)	Bacteria (commercial probiotic)	Protexin® (*E. faecium*)	Ingestion	No addition	(+) Increased survival (+) Reduced pathogen loads	NA	[73]
Insect, honey bee (*A. mellifera*)		Bacteria (consortium)	*Snodgrassella alvi*, *Gilliamella apicola*, *Bifidobacterium asteroides*, and *Lactobacillus* nr. *melliventris*	Ingestion	Placebo	(+) Increased survival	Yes	[71]
Coral (*Mussismilia harttii*)	Remediation of oil spills	Bacteria (consortium)	*Bacillus rigui, Acinetobacter calcoaceticus, Bifidobacterium catenulatus/indicus/cibi, Bacillus aryabhattai, Paracoccus homiensis, Paracoccus kamogawaensis, Paracoccus marcusii, Psychrobacter* sp., *Vibrio alginolyticus*, and *Pseudomonas stutzeri*	Administration in surrounding environment	No addition	(+) Increased photosynthetic efficiency (+) Increased calcification biomarkers (–) Increased lipid peroxidation	Yes	[23]
Coral (*Acropora tenuis* and *Platygyra daedalea*)	Assess feasibility of coral microbiome manipulation in early life stage	Bacteria (consortium)	*Acinetobacter, Bacterioplanes, Marinobacter, Paracoccus, Pseudoalteromonas, Pseudovibrio*, and *Vibrio*	Administration in surrounding environment	Placebo	NA	Yes	[97]
Coral (*Pocillopora damicornis*)	Mitigation of heat stress/bleaching combined with pathogen challenge (*Vibrio coralliilyticus*)	Bacteria (consortium)	Five *Pseudoalteromonas* spp., *Halomonas taeanensis*, and *Cobetia marina*	Topical administration	Placebo	(+) Increased photosynthetic efficiency (+) Reduced bleaching	Yes	[20]
Coral (*Acropora millepora*)	Mitigation of heat stress/bleaching	Dinoflagellate (single)	*Durusdinium trenchii* and *Cladocopium goreaui*	Administration in surrounding environment	Placebo	(+) Increased survival (+) Reduced bleaching (+) Increased photosynthetic efficiency	NA	[101]
Coral (*Mussismilia hispida*)	Mitigation of heat stress/bleaching	Bacteria (consortium)	*Bacillus lehensis, Bacillus oshimensis, Brachybacterium conglomeratum, Planococcus rifietoensis*, and *Salinivibrio* sp.	Topical administration	Placebo	(+) Increased survival (+) Increased photosynthetic efficiency (+) Upregulation and downregulation of key cellular processes (+) Restructured metabolome	Yes	[15]

(continued on next page)

Table 1. (continued)

Host	Aim of probiotic application (in case of diseases, details on pathogen are given in parenthesis)	Type of probiotic (details on the use of single species or consortia are provided in parenthesis)	Probiotic species	Probiotic application	Type of control	Effect of probiotic on measured host variables: (+) positive effect (=) no difference between treatments (–) negative effect	Microbiome changes	Refs
Coral (*Millepora alcicornis*)	Remediation of oil spills	Bacteria and fungi (consortium)	*Halomonas aquamarina, Pseudoalteromonas shioyasakiensis*, two *C. marina, Shewanella* sp., *Ochrobactrum anthropi, Rhodotorula mucilaginosa, Geotrichum* sp., and *Penicillium citrinum*	Administration in surrounding environment	Placebo	(+) Increased photosynthetic efficiency	Yes	[38]
Coral (*P. damicornis*)	Improvement of physiological parameters	Bacteria (consortium)	*Yangia, Roseobacter, Phytobacter*, and *Salinicola*	Administration in surrounding environment	Placebo	(+) Increased energy reserves (protein, lipids, and carbohydrates) (+) Increased calcification (=) Pigments and photosynthetic efficiency	Yes	[102]
Plant, clover (*Trifolium repens*)	Increase of tolerance to heavy-metal-polluted soil	Bacteria and fungi (single and consortium)	*Brevibacillus* sp. and *Glomus mosseae*	Administration in surrounding environment	Dead/denatured cells	(+) Increased plant growth (+) Increased arbuscular mycorrhizal colonization (+) Increased nutrient acquisition (+) Reduced metal uptake	NA	[83]
Plant, legume (*Retama sphaerocarpa*)	Increase of drought tolerance	Bacteria and fungi (single and consortium)	*Bacillus thuringiensis* and *Glomus intraradices*	Topical application and administration in surrounding environment	Dead/denatured cells	(+) Increased root growth (+) Reduced water requirement	NA	[81]
Plant, clover (*T. repens*)	Increase of tolerance to heavy-metal-polluted soil	Bacteria and fungi (single and consortium)	*Bacillus cereus* and *G. mosseae*	Administration in surrounding environment	Dead/denatured cells	(+) Increased plant growth (+) Increased arbuscular mycorrhizal colonization (+) Increased nutrient acquisition (+) Increased antioxidant activities (+) Reduced metal translocation	NA	[82]
Plant, clover (*T. repens*)	Increase of tolerance to heavy-metal-polluted soil	Bacteria and fungi (single and consortium)	*B. cereus, Candida parapsilosis*, and *G. mosseae*	Administration in surrounding environment	Dead/denatured cells	(+) Increased plant biomass (+) Increased arbuscular mycorrhizal colonization (+) Increased pollutant tolerance	NA	[84]

[a]Abbreviation: NA, not applicable.

example, a higher leukocyte count was found in the recipients [129]. Microbial debris and products filtered from microbial cells have also been efficiently tested as a promising alternative microbial therapy that does not rely on living cells [130]. The use of postbiotics instead of probiotics can be attractive, for example, in terms of shelf-life and safety, especially considering immunocompromised individuals [111,113,117]. However, the efficacy of each of these treatments is variable [112,113] and could be a key component in the selection of the 'best' microbial therapy. Therefore, use of dead or live cells should be considered as two different paths of different microbial therapies that will offer optimal outcomes that can be applied in different contexts/situations, depending on the experimental goals, logistics, and expertise.

Accounting for nutritional benefits of probiotic cell administration

Gauging the mechanisms underlying probiotic effects (or other microbially mediated benefits) in wildlife is challenging. In most agriculture and aquaculture production systems, diets are supplied in excess, and probiotic supplements do not provide any significant nutritional benefit. By contrast, some authors argue that the supplementation of probiotics in other systems, such as corals, introduces the possibility that observed benefits can also be attributed to a direct nutritional effect [98]. For instance, while Morgans and collaborators [101] found an unequivocal benefit of inoculation with a dinoflagellate probiotic candidate, the strain was not detected in the tissue of the recipient host after inoculation. In this case, the placebo was insufficient to rule out the inoculated cells' nutritional value or postbiotic effect. Therefore, whether the observed benefits could have resulted from a combination of other specific confounding factors (Figure 1), including a nutritional benefit, induction/restructuring of the associated microbiome, or a response induced by specific metabolites (i.e., postbiotics) added with the inoculation, remains unresolved.

The density of probiotic cells applied in inoculations is an important factor that needs to be considered to better understand whether a probiotic effect can be explained by its nutritional value. For example, cell densities applied in inoculations of corals using dinoflagellates (3×10^4 to 1×10^6 cells/ml) [101,131,132] exceed those reported from natural algal blooms [133] or implemented in coral feeding experiments [134] by up to 1000-fold, hence projecting the possibility of a nutritional effect. Prokaryotic probiotics, however, are used as single or few inoculations at densities below 1×10^4 to 1×10^6 cells/ml [15,20,23,97,102], which is comparable with, or lower than, bacterial cell densities in natural reef water [135]. These probiotic additions would be negligible from a nutritional point of view. The possibility of a nutritional effect is likely even lower in field studies, in inherently open systems, which will dilute the concentration of probiotics further.

Tracing the fate of probiotic cells

Colonization is not a requirement for probiotic efficiency [136,137] as microbes can also promote health by triggering host immune responses and microbiome restructuring or through probiotic effector molecules, including cell membrane proteins, polysaccharides, or bacterial metabolites [15,136,137]. Indeed, the very fact that microbiomes are usually flexible in nature and can vary depending on different environmental conditions and/or anthropogenic stressors – which has been shown in coral transplantation experiments [138] – is also a premise for the use of probiotics as a means to restore microbiomes [24]. Their tendency to return to their original assemblages also supports probiotics' safety when administration or enrichment of probiotics cease [137]. Despite this eventual temporary nature, the improvements provided by the application of probiotics can contribute to the survivorship of the recipients through stress events, retaining the biodiversity until more permanent solutions are achieved [26,139].

When colonization is achieved (which may be more likely when native bacteria are used), at least temporarily, the tracing of the uptake and retention of probiotics can provide additional insights

into their beneficial activity and localization within the host. Diverse approaches have been utilized for tracking probiotic cells within recipient microbiomes, with applications across various host types. For example, amplicon sequence techniques have been used to detect the bacterial taxa that could have been transmitted or enriched in the recipient through the microbiome transplant method for corals [33]. qPCR amplification methods have been used to detect the antimicrobial activity of probiotic formulation in humans and for the quantification of probiotics in poultry feed and gut [140,141]. Epifluorescence microscopy and fluorescence *in situ* hybridization techniques have been utilized to visualize probiotic cells [142]. Whether the colonization and/or effect of probiotics will be short-lived or persist for the life of the individual needs to be investigated in long-term studies. Short-lived probiotic activity would allow for applications where a short-term physiological gain is advantageous, such as pathogen resistance during an outbreak or stress tolerance during exposure to environmental stress (e.g., acute heat waves or the presence of chronic local stressors). Microorganisms associated with corals might exert a direct or indirect influence on the phenotypic response of the coral by modulating its epigenome [143]. Nevertheless, a persistent association with probiotics or long-lasting effects through microbiome restructuring or epigenetic changes [143] may also be desirable for long-term resistance to impacts. Future studies should address this knowledge gap and establish suitable protocols for long-term tracking of probiotics and their activity in wildlife.

Identifying the probiotic mechanisms of action

An ultimate verification of specific probiotic mechanisms of action lies in the use of gene-editing technologies on probiotic cells or communities. For example, recombineering, deletion of genes, or CRISPR-based systems [144] can be used to create functional knockouts that are devoid of the production of a putatively active molecule. However, it is important to note that beneficial effects may be multifactorial and, likely, one knockout treatment may only explain a fraction of a whole beneficial effect. Nevertheless, this approach could be advantageous by allowing for a systematic approach to understanding specific mechanisms. If a reduction in the probiotic-driven protection correlates with the depletion of a specific mechanism, such a trait could be considered to be, at least, one of the 'probiotic factors'. Often, these efforts require previous foundational knowledge of the metabolic pathways involved and the tools to inactivate them. Obtaining this information is often a challenging task, especially for nonmodel organisms. In such cases, however, 'omics'-based studies can effectively identify host transcripts that are significantly upregulated and downregulated by probiotic inoculation and correlate with health improvements, which can assist in tracking down mechanisms of action [15] and identify promising new probiotic candidates [145]. Although the advancement of deciphering mechanisms-of-actions of probiotics is challenging, these efforts are worthwhile as they will help optimize probiotic applications.

Administration and scaling up production of probiotics for wildlife

In addition to optimizing the production at laboratory or industrial scales and the viability of cells, the delivery method for probiotic administration [146] must be considered in product development.

Probiotic administration strategies are contingent upon the host, its environment (aquatic or terrestrial when considering wildlife), and the treatment goal (e.g., amelioration or prevention of skin disease in amphibians or enhancement of thermal tolerance in corals). So far, in most wildlife studies, probiotics were applied directly as cell suspensions in the surrounding environment, such as inocula in coral aquaria [15,20,102], mixed with food provided to tadpoles [93] and bees [74], or directly on the host skin of amphibians [51,77] and bats [69]. Once a probiotic

has been proven effective, research efforts should focus on the development of delivery strategies that reduce its dispersion in the environment, ensure delivery, minimize effects on nontarget organisms, and reduce costs that together can scale up probiotic applications to natural populations both on land and in the sea [24,49,147,148].

Depending on the nature of the probiotic action and the host, several strategies can improve the successful delivery in natural populations, including probiotic encapsulation in live feed [148], when ingestion is the best delivery strategy or immobilization for slow release of probiotics to the environment. In fish aquaculture, early delivery at the larval stage through cell enrichment in the culturing environment for pre-feeding larvae has been shown to increase the incorporation and retention success of the probiotic later on [149]. Hence, the time point of delivery (e.g., host life stage), 'packaging' of probiotics, and delivery method all deserve careful consideration.

Production and formulation of microbes at a large scale is another major challenge in probiotic development, especially for nonmodel microorganisms [29]. Scaling up from laboratory production – of the order of liters to hundreds of thousands of liters for industry-scale production – requires optimization and standardization of growth protocols and specialized equipment and installations. Preservation methods (e.g., freezing or lyophilization), which enable the proper storage and transportation of cells without compromising their viability, are also important to consider. Ensuring cell viability is therefore crucial not only during the large-scale production of probiotics but also for their formulation and storage of products. Teaming up with industries and laboratories specialized in the large-scale production and long-term preservation of probiotics, such as those commercialized for human consumption, is, for example, an alternative to boost such development.

Safety

The logistics, speed, and costs of scaling-up probiotic usage are big emerging challenges in the implementation of probiotics for wildlife. Another top priority is to minimize nontarget effects. A science-based framework was recently proposed to ensure the ethical and careful stewardship of wildlife and environmental microbiomes, detailing necessary risk assessment steps to guide such studies [14]. Briefly, these steps include an initial case-by-case assessment and a preference for the bioaugmentation of native and/or commonly abundant probiotic cells. Other crucial steps are the exclusion of any potential pathogens, the use of probiotic dosages that are comparable with natural concentrations of these microorganisms, and the evaluation of potential risks versus the benefits of the use of probiotics for the target organism and its environment [14].

Alternative microbe-based therapies

We highlight that expanding the tested approaches and testing **alternative microbe-based therapies**, such as the use of prebiotics [150], postbiotics [124], or microbiome transplantation [151,152], can also help advance the field. Microbiome transplantation, for instance, has long been part of clinical routines and agricultural applications to treat diseases and enhance health, productivity, and stress tolerance of humans and other organisms [22,153] and can be applied long before ready-made probiotics are available and the microbial solutions to a given problem are elucidated. It has been proposed as a tool for wildlife conservation [154], while the first experimental trials have already been performed in corals and koalas [33,155]. Investigations of this method can be used to expand our knowledge on probiotic microbes that are difficult to obtain as pure cultures. Also, prebiotics, utilized as a strategy for the prevention of infectious disease in the food production sector, for example, aquaculture [156], are slowly finding their way into wildlife conservation and protection of critically endangered species, as recently exemplified in the recommendation to use carotenoids for microbiome enhancement to modulate host

resistance in the threatened Southern Corroboree frog species [157]. To experimentally validate these alternative applications, the same recommendations apply, as outlined earlier. Exploring such broader and less-targeted strategies of microbiome manipulation might reveal some of them as suitable to advance our knowledge of microbe–host interactions, which may help optimize probiotic and other methods and allow microbiome restoration, for example, in organisms where microbiomes are complex and specific beneficial microbes are not yet identified [158].

A roadmap for studies of emergent probiotics for wildlife and alternative microbe-based therapies

Based on the discussion and examples provided earlier, we suggest a basic and robust roadmap (Figure 2) describing priorities and alternatives in the development of probiotics for wildlife, including the optimization and scaling up delivery of probiotics and, where possible, elucidating their underlying mechanisms, which should be helpful to boost future developments of probiotic treatments for wildlife. We propose the following steps:

(1) Priority – culturing and selecting probiotics:

Strong efforts are needed to implement more cultivation-based approaches into microbiome research as well as understanding of keystone species and their selection.

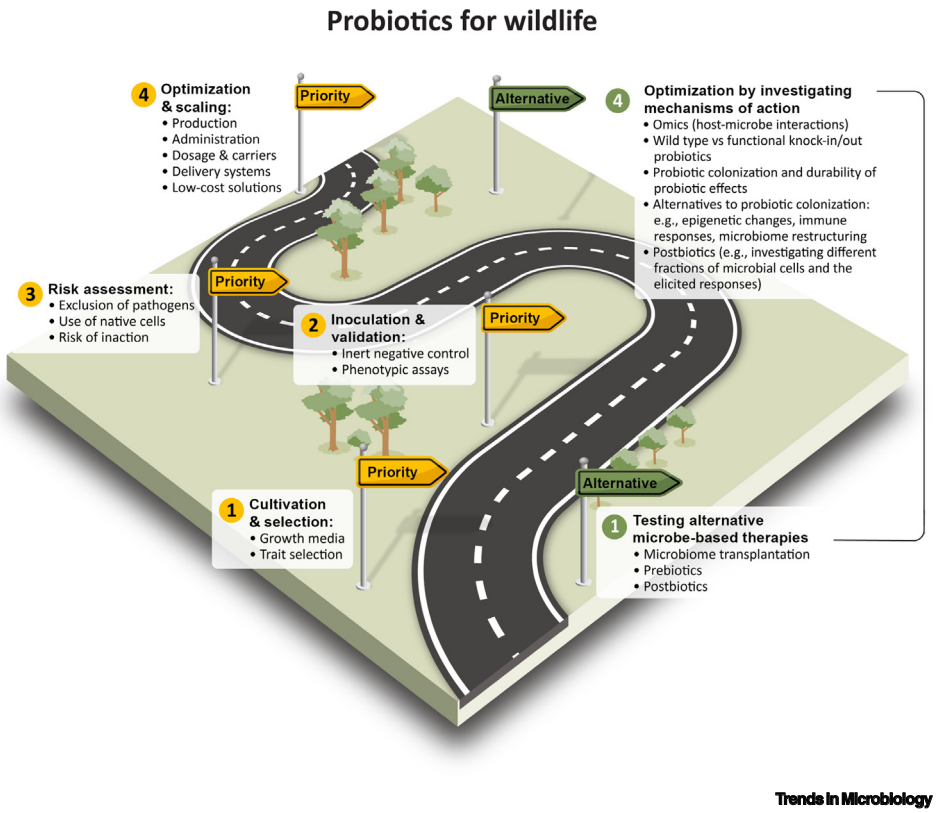

Figure 2. Roadmap of challenges and opportunities in the development of probiotics for wildlife. Basic requirements and opportunities to advance our knowledge and selection of probiotics for wildlife, as well as their improvement and implementation. Additional experiments to explore alternative tools (i.e., postbiotics) and protective mechanisms (e.g., nutrition, postbiotic, probiotic) may increase our knowledge and improve the use of microbial-based therapies.

Alternative – Testing alternative microbial therapies: Testing alternative microbe-based therapies, such as the use of postbiotics or microbiome transplantation, can help to identify mechanisms of protection.

(2) Validating probiotic effects:

An inert negative control (ideally a placebo) is essential for validating and quantifying probiotic effects on recipients, accounting for basic confounding effects related to handling procedures and environment.

(3) Risk assessment:

Follow the science-based framework previously proposed, focused on using ethical guidelines, excluding pathogens, optimizing probiotic dosage, prioritizing the bioaugmentation of common, native microbial cells, and taking into account the risk of inaction [14,66]

(4) Priority – optimization and scaling

Investigations into range-finding for optimal dosages and carriers for probiotic inoculation should be included in research agendas, including the search for low-cost solutions that are easy to manipulate and deploy at scale.

Alternative – Optimization through elucidation of mechanisms of action:

- The use of omics to pinpoint specific mechanisms of action, or experimenting with probiotic homogenates [159], supernatants [160], or fractions of probiotic extracts [130].
- The use of functional knockin/out microbes (in comparison to their wild-type) and 'omics'-based surveys are also strongly encouraged for research purposes, particularly when the elucidation of a specific probiotic mechanism is one of the research goals.
- Tracing probiotic enrichment, microbiome restructuring, mechanisms of protection and/or incorporation in the recipient organism can decipher between the need for probiotic colonization or the presence of alternative probiotic effects (such as triggering immune responses, epigenetic changes, or microbiome restructuring).
- The testing of additional treatments, such as exposure to dead cells (i.e., postbiotics) is encouraged, as in some cases they may represent an easier, safer, and still efficient alternative application or help to elucidate mechanisms involved with microbial protection.

Insights on the mechanism will eventually feed back into the optimization of the methods and help in the design of administration strategies as well as scaling-up probiotic applications.

Concluding remarks

Probiotics are already contributing to performance and health improvements in different organisms [6,7,21,44], including crops, livestock, aquaculture species, and humans. Currently, a growing research focus is the development of probiotics to help address ecological crises and biodiversity losses, such as the degradation of agricultural lands or the decline of threatened species including corals, amphibians, bees, and bats [8,14–16,20,48]. Taking probiotics from controlled and relatively small-scale environments, such as agricultural fields, aquaculture facilities, or human bodies, to native animal populations and ecosystems comes with new challenges [161]. Among these, the spatial and temporal scales over which the probiotics need to provide a benefit to host organisms, and the variable environmental conditions under which this needs to be achieved, are some of the biggest hurdles. Hence, further knowledge is needed to fill current

Outstanding questions

What is the current state-of-the-art use of probiotics for wildlife?

What is the best possible validation practice (i.e., use of negative controls) for probiotic studies?

What are the main challenges, and what can we learn from established 'routine' uses of probiotics and other microbial therapies to accelerate applications for wildlife?

How can we increase our knowledge of the mechanism(s) underlying probiotic effects and use it to improve the efficacy of probiotics?

gaps in understanding the mechanism of action of probiotic candidates and how to best apply, test, and track probiotics in nature [14]. Robust experiments that include a placebo control group, which have successfully paved the way for effective probiotics used today by industry and clinicians, are strongly recommended to detect the efficacy of emerging probiotic applications. Experimental use of knockin/out microbes (developed at the laboratory scale) and the comparison of promoted probiotic effects with their wild type, can, for example, support the identification of specific probiotic mechanisms, accelerating and improving the selection of additional probiotic candidates. Furthermore, 'omics'-based research can also contribute to the elucidation of beneficial mechanisms without the use of genetic manipulation [6,15,106,109,145]. Similarly, protocols for the development of alternative microbial-based therapies that have been tested for other better-studied organisms (e.g., humans and plants), such as microbiome transplants and postbiotics (i.e., inactivated cells) [22,30,32,121,153], may aid the search for effective and scalable microbial therapies to counter the current loss of biodiversity.

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Declaration of interests

No interests are declared.

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