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REVIEW

Phage lysins for intestinal microbiome modulation: current challenges and enabling techniques

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ABSTRACT

The importance of the microbiota in the intestinal tract for human health has been increasingly recognized. In this perspective, microbiome modulation, a targeted alteration of the microbial composition, has gained interest. Phage lysins, peptidoglycan-degrading enzymes encoded by bacteriophages, are a promising new class of antibiotics currently under clinical development for treating bacterial infections. Due to their high specificity, lysins are considered microbiome-friendly. This review explores the opportunities and challenges of using lysins as microbiome modulators. First, the high specificity of endolysins, which can be further modulated using protein engineering or targeted delivery methods, is discussed. Next, obstacles and possible solutions to assess the microbiome-friendliness of lysins are considered. Finally, lysin delivery to the intestinal tract is discussed, including possible delivery methods such as particle-based and probiotic vehicles. Mapping the hurdles to developing lysins as microbiome modulators and identifying possible ways to overcome these hurdles can help in their development. In this way, the application of these innovative antimicrobial agents can be expanded, thereby taking full advantage of their characteristics.

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Introduction: the gut microbiome and microbiome modulators

The gastrointestinal tract of humans is inhabited by a variety of microorganisms, including bacteria, viruses, archaea, fungi, and protozoa. These microorganisms are of paramount importance for the physiological functioning and health status of the human host, as reported by an accumulating number of studies.[1,2] Both entities have acquired certain functions to adapt to each other, and they are even considered to have coevolved.[3] This has resulted at some levels in symbiotic relationships. This symbiosis can be mutually beneficial but can also be neutral or even disadvantageous (also called pathogenic) to the human host.[3] In any case, the continuous interplay between the human host and microbiota aims to achieve a harmonious, homeostatic state.[1] During this homeostatic state, the microbiota provides certain vital functions to the host.[3] These include regulation of digestion[4] and immune response,[5] suppression of pathogens,[6] and maintaining epithelial integrity.[7] However, a disruption of the homeostatic state, also termed dysbiosis, is associated with multiple diseases and disorders, including *Clostridioides difficile* infection,[8] inflammatory bowel disease,[9] celiac disease,[10] colorectal cancer,[11] obesity,[12] and diabetes.[13]

The associations of the microbiota with health and disease, together with the important functions it provides, have resulted in a paradigm shift in how to perceive the role of the microbiota for human health and its implications for healthcare.[14] Two main drivers can be identified. On the one hand, broad-spectrum antibiotics are questioned in some cases, not only because the target bacteria are becoming resistant to them, but also because they disrupt the normal microbiota, with effects observed in the short and the long term.[15–17] This, in turn, can have detrimental effects on the human host, although the target bacterium and primary infection can normally be treated.[15] Antibiotic-associated diarrhea and

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a bloom of vancomycin-resistant enterococci upon antibiotic treatment are two examples of such effects in the short term.[18] Furthermore, although association should not be confused with causation, the use of broad-spectrum antibiotics in early childhood is associated with obesity later in life. It is thought that the long-term effects of antibiotic treatment on the human host may be caused by a disruption in the establishment of homeostatic microbial colonization.[19,20] Anyway, the off-target and potentially long-term effects of broad-spectrum antibiotics have led to an increased interest in narrow-spectrum antibiotics that kill or inhibit the growth of the target bacteria while keeping the rest of the microbiota intact.[21] To use these antibiotics effectively, precise and rapid diagnostics are required to identify the target bacterium and the accompanying antibiotic.[22] On the other hand, the association of the microbiota with health and disease has sparked interest in steering the intestinal microbiota to treat certain diseases by altering the microbiota composition or adding certain functions to the microbiome. For example, fecal microbiota transfer has been considered as a treatment by remodeling the gut microbiota, with promising outcomes for treating *C. difficile* infections, but variable outcomes in the case of inflammatory bowel disease.[23,24] These insights have led to a paradigm shift in the perception of bacteria in healthcare: (i) encouraging results of treating diseases by remodeling the gut microbiota and (ii) the association between the adverse effects of broad-spectrum antibiotics on the gut microbiota and human health.

Because microbiome research is constantly and rapidly evolving, some terms used in this article are clarified to avoid confusion. The vast number of living microorganisms in the intestinal tract, of which bacteria alone make up an estimated 39 trillion microorganisms, is collectively referred to as 'the human intestinal microbiota'.[3,25] This human intestinal microbiota, together with the collective genome and the produced metabolites, controlled by environmental factors is called the 'human intestinal microbiome'.[3] It is difficult, if not impossible, to properly define what a healthy microbiome is, as microbiomes vary between healthy individuals, and thus a healthy microbiome is not necessarily determined by the presence of one or some specific microbial species. At most, it can be said that a holobiont is characterized by a healthy homeostatic state with high microbial functional diversity and a harmonic microbial balance. However, an absolute, general definition of this 'balance', also called 'eubiosis', cannot be given, also not for the counter situation, 'dysbiosis' in which the homeostatic balance is lost.[26,27] Due to the relative and variable nature of a healthy microbiome, it is also difficult to provide a definition of 'microbiome-friendliness' as used in the field of antimicrobial therapy or microbiome modulation. Still, some attempts have been made, arguing that this notion should be characterized as a lack of microbiome perturbation with respect to a basal, homeostatic state. In this sense, the eradication of opportunistic pathogens and stimulation of 'beneficial' microbes are perceived to contribute to a microbiome-friendly character, although both effects can be seen as compositional changes.[28] As it can be concluded from our prior discussion, defining which organisms are opportunistic pathogens and which are beneficial is a challenging task, if *bona fide* beneficial microorganisms even exist. In this regard, advances in microbiome research might help in elucidating which species may be regarded as key toward promoting a healthy microbiome in general, or if this issue needs to be assessed in a case-by-case manner, providing elements for more concrete definitions of the microbiome-friendliness of microbiome modulators and antimicrobial agents.[29] What we refer to as 'microbiome modulation' are techniques aiming to change the composition of a microbiome to restore its homeostatic state. This artificial modification of the microbiome composition can be achieved by expanding the presence of certain species, for example, by fecal microbiota transplantation,[23] by using pre-,[30] pro-,[31] or postbiotics,[32] or by the administration of a microbial consortium.[33] Conversely, the microbiome can also be modulated by eliminating species or strains that are considered to provoke negative effects. Although broad-spectrum antibiotics are usually administered to diminish disease-causing bacteria in situations of dysbiosis, their effect on other gut bacteria can also hinder the recovery of a homeostatic state. Therefore, a more specific depletion of species or strains is considered to be a more suitable way of

microbiome modulation. This can be achieved, for example, (i) by using narrow-spectrum antibiotics,[34,35] (ii) by using bacteriophages that infect and kill specific bacteria,[36,37] (iii) by engineering bacteria that produce or deliver antimicrobials and toxins targeting nearby bacteria,[2,38–41] and (iv) by delivering genetic tools to eliminate target species, *e.g.* the selective destruction of target DNA by CRISPR-Cas systems.[42–44] For a more comprehensive review of the impact of the microbiome on human health, potential engineering techniques, and enabling technologies, the reader is referred to Aggarwal et al. (2023).[2] In this work, we particularly focus on the use of phage lysins as potential microbiome modulators.

Phage lysins as a potential tool for microbiome modulation

Many bacteriophages, viruses infecting bacteria, encode lysins, enzymes that catalytically degrade the peptidoglycan of the bacterial cell wall. Their natural role is to lyse the cell at the end of the replication cycle, resulting in the release of the newly formed phage particles. Most phage lysins from Gram-positive infecting phages are modular, meaning they consist of one or more modules or domains, folding and acting independently as either an enzymatically active domain (EAD) or a cell wall-binding domain (CBD). In contrast, most lysins targeting Gram-negatives are globular, consisting of a mere EAD. For a comprehensive review of phage lysins, the reader is referred to.[45] In the case of Gram-positive bacteria, purified wild-type phage lysins can remain active when applied exogenously, resulting in osmotic lysis and subsequent cell death. They can thus be used as enzyme-based antibacterials. These lysins are considered a promising new class of antimicrobials because they have a new mode of action, a low probability to provoke resistance development, a lack of cross-resistance with known resistance mechanisms, and a new target as compared with the standard-of-care antibiotics. In addition, they act rapidly and may have a narrow spectrum. Moreover, the modular nature of lysins enables relatively easy protein engineering by domain swapping.[46] For the sake of clarity, in this review, phage lysins and engineered, phage-derived lysins are taken under the umbrella term 'lysin'. The protein engineering process allows the improvement of phage lysins by altering their bactericidal activity and biochemical properties, modifying the lytic spectrum, or improving the performance of the protein in clinical settings.[47] Moreover, the activity spectrum can be further tuned by domain swapping. This is a particularly appealing property in terms of increasing the specificity of microbiome modulation. Indeed, phage lysins have the potential to act as precision antibiotics that surgically remove the pathogen, without or with minimal effect on the benign microbiota.[46] This becomes increasingly relevant now it is increasingly understood that the collateral damage of widely used broad-spectrum antibiotics on the intestinal microbiota causes dysbiosis, which is associated with multiple diseases.

The implementation of phage-derived lysins to treat bacterial infections while maintaining or restoring a healthy microbiome has gained interest of the industry. For example, lysin SA.100 is incorporated in the cosmetic product Gladskin to treat skin diseases aggravated by *S. aureus*. Moreover, other lysins are being developed to selectively eradicate *S. aureus* in the skin microbiome, lysin XZ-700 being one example.[48–50] The possibilities of an engineered lysin, PM-477, has also been explored to treat bacterial vaginosis by specifically eradicating *Gardnerella* while having limited impact on beneficial lactobacilli or other species in the vaginal microbiome.[51] Medolysin®, a burn wound spray that eradicates either Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* or Gram-positive *S. aureus*, using the Artilysin® technology. This technology is based on the fusion of a modular lysin with a lipopolysaccharide-destabilizing peptide. Medolysin® has been reported to have positive effects on the skin microbiome; however, no data supporting this claim are available.[52,53] In addition to Medolysin®, other Artilysins® are being developed to treat skin diseases aggravated by bacterial infections while allowing the skin microbiome to be restored.

The academic research and commercial investments in lysin technology point toward the feasibility of using them as microbiome-friendly agents to modulate microbiomes. This entails that they could specifically deplete bacteria that cause adverse effects to the host, while generally keeping the benign bacteria of the microbiome intact.

Because lysins directly act on a target bacterium, they are not dependent on the presence or proliferation of certain bacteria, as is the case for, for example, pro-, pre-, and postbiotics, fecal microbiota transfer and engineered bacteria specifically delivering a toxin or antimicrobial substance. Moreover, because lysins actively degrade the cell wall of the target bacteria rapidly, the metabolic status of the target cell has only a small effect on its action, if any. This counteracts the evasion of lysin treatment by bacterial persisters (bacteria with the same genotype that are metabolically inactive)[54] and resisters (bacteria with new genotype that are not affected by the treatment).[49,55] In contrast, antibiotics and bacteriophages require an active metabolism of the target bacteria. This, in turn, can result in the survival of persisters that can repopulate the infection site upon resuscitation,[56] and promote the emergence of resisters,[57,58] interfering with the complete eradication of the target bacterium.

Although lysins are promising candidates to modulate the gut microbiome, the current applications are limited to treating skin and vaginal infections. These microbiomes are usually less complex, having an estimated lower absolute amount of bacterial cells (estimation of 10^{14} bacteria in the colon and 10^{11} in both the vaginal and skin microbiota[59]) and a lower (in case of the vaginal microbiome) or similar (in case of the skin microbiome) alpha diversity (measure of microbial diversity within a sample) compared to the intestinal tract.[60] Moreover, the application of products is easier for skin and vaginal applications, compared to the intestinal tract.[2] In this review, challenges encountered when developing phage-derived lysins to modulate the gut microbiome, including defining the specificity profile and microbiome-friendliness, and the delivery to the gut, as well as potential enabling techniques are explored (Figure 1).

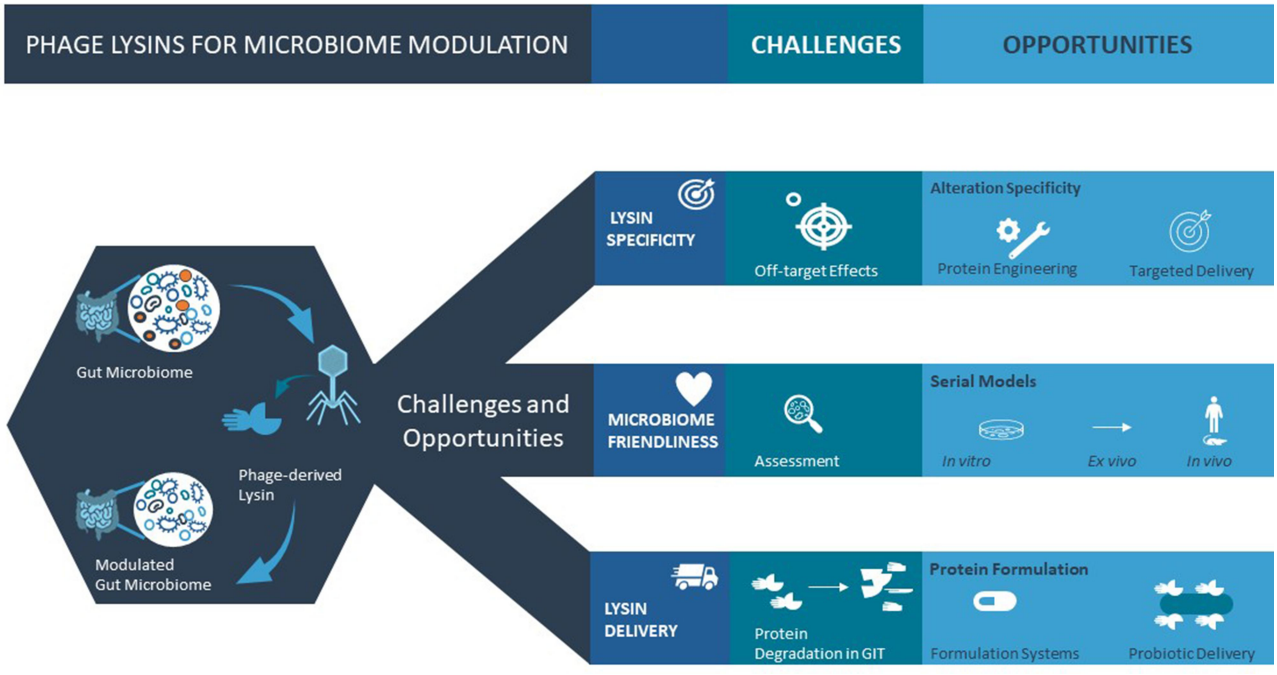

Figure 1. Phage-derived lysins are potential microbiome modulators. However, certain challenges hinder their development, which could be addressed by recent opportunities. (left) the microbiome within the gut can be altered and modulated by using lysins derived from phages. After treatment with a phage-derived lysin, the gut microbiome is depleted from specific members. (upper right) the specificity of lysins can be too broad, resulting in off-target effects. Protein engineering and targeted delivery of lysins can be applied to minimize these effects. (middle right) assessing the impact of a lysin on the microbiome can be challenging, partly due to the complexity of the microbiome and ethical issues. Therefore, consecutive *in vitro*, *ex vivo* and *in vivo* assessment models could be applied. (lower right) lysin delivery to the intestines is complicated because proteins can be degraded during passage through the gastrointestinal tract (GIT). Proteins can be formulated using probiotic delivery methods, particle-based and macroscopic systems to address this issue. GIT: gastrointestinal tract.

Challenges and enabling technologies

Specificity of lysins

Lysins are considered microbiome-friendly because of their specificity. Indeed, specificity can give an interesting first insight into lysin behavior and can be considered a first step toward the characterization of a lysin and its potential as a microbiome modulator with a microbiome-friendly character. Lysin specificity is usually examined by assessing the lytic or bactericidal activity against a panel of bacteria. This panel is usually made up of closely related species, sometimes complemented by species relevant to a given microbial environment.[51] However, the selection of a panel of bacteria may seem arbitrary because defining species relevance for a given microbiome is not straightforward. In this regard, ongoing efforts to characterize the human microbiome in health and disease, fueled by advances in sequencing and culturing technologies, can help in the selection of a relevant panel.[61,62] Besides the choice of a bacterial panel, the conditions in which the lysin has been tested are also of major importance. Most antibacterial assays performed to test lysin specificity are executed under laboratory conditions, using one specific strain at a time under controlled conditions such as pH, temperature, *etc*. Although these conditions increase the reproducibility and robustness of experiments, they do not consider the complexity of the intestinal environment. As bacterial growth kinetics, cell morphology and associated peptidoglycan structure, and bacterial functionality can vary across different growth conditions and environments, this can affect lysin activity.[63,64] In addition, lysin activity itself depends on environmental conditions, such as ionic strength and tonicity.[65] Taken together, assessing the specificity of a lysin is an important first step toward characterizing it as a microbiome modulator, but care should be taken when choosing which conditions and which bacteria are to be examined.

That being said, it is important to note that not all lysins exhibit the same level of specificity. While some lysins are indeed reported to possess a specific bactericidal activity, often at the serovar, species,[66] or genus level,[67,68] others exhibit bactericidal activity against a broader range of bacteria.[69–72] The differences in lysin specificity could be partly attributed to its modular architecture and its target. However, not all lysins are modular. In fact, most Gram-negative lysins are monomodular or globular.[73] Notably, although generalizations should be taken with caution, lysins from Gram-negative phages appear to have a broader host range compared to those from Gram-positive phages. This can be explained on the one hand by the importance of the CBD in determining lysin specificity, although evidence suggests that the EAD also contributes to lysin specificity, and on the other hand by the conserved peptidoglycan chemotype (A1γ) for Gram-negatives, whereas Gram-positive bacteria show much more chemotype diversity.[74–76] In any case, it can be said that lysins have, in general, a broader host range compared to their encoding phages, but are still more specific than currently used broad-spectrum antibiotics.[77,78] To further fine-tune the specificity of lysins, it is possible to adapt lysin specificity using different approaches, including protein engineering and directed delivery.

First, the specificity of a lysin can be altered using protein engineering. Swapping EADs and CBDs originating from different lysins is a viable approach to alter the specificity or other characteristics. This has been applied to broaden, narrow, and even redirect the activity range of lysins.[47,79] For example, the domains of two enterococcal lysins with different lytic specificities, PlyV12 and LysEF-P10, were swapped to modulate their specificity. By substituting the CBD of PlyV12 with that of LysEF-P10, the lytic activity of PlyV12 was narrowed, targeting only enterococci. On the opposite, the substitution of the CBD of LysEF-P10 with that of PlyV12 broadened the activity range of LysEF-P10, targeting *E. faecalis* and staphylococci, but not *E. faecium*.[79] Similarly, the fusion of the CBD of PlyV12 with the EAD of *Staphylococcus aureus* phage lysin LysGH15 or the EAD of bacteriophage endolysin Ply187 extended the activity spectrum toward staphylococci, enterococci and streptococci.[80,81] Likewise, ClyR, constituting of the CBD of *Streptococcus suis* prophage lysin PlySs2 and the EAD of C1 bacteriophage lysin PlyC, showed extended lytic activity against streptococci, enterococci and staphylococci.[82] In addition to domain swapping, domains have been truncated or deleted,[83–85] or mutagenesis has been employed to alter lysin properties.[86–89] Moreover,

lysins or lysin modules can be fused to other peptides or protein moieties.[90] The latter approach is mainly applied to enable lysins to pass through outer or eukaryotic membranes to potentiate lysins against Gram-negative and intracellular bacteria, respectively, but it is also used to alter other properties, such as directing lysins toward specific targets. For example, fusion of LysP (the endolysin of *Propionibacterium* phage P1.1) to a cellulose-binding domain enabled cellulose-based affinity protein purification[91]; fusion of the enzybiotic Cpl-711 to MinP (a minimized polyhydroxyalkanoate affinity tag) enabled lysin immobilization on a polymeric nanoparticle,[92] and fusion of a chimeric lysin (Cpl-7S) to a choline-binding module was performed to redirect the chimeric lysin toward choline or its derivative diethylaminoethyl.[93] In conclusion, the fusion of lysin domains to a plethora of possible peptide moieties could be applied to modulate lysin characteristics, including redirecting lysins toward a specific target.

A second possible approach to enhance lysin specificity is to target their delivery toward a specific cell type. This could be achieved, for example, by engineering (probiotic) bacteria in such a way that they produce or secrete a drug upon the detection of the target bacteria, by means of quorum sensing molecules, bacterial pheromones, or molecules characteristic to a specific disease state. For example, Art-085 was constitutively expressed in *E. coli*, and released upon detection of a quorum sensing signal of *Vibrio cholera*.[39] Besides phage-derived lysins, other antimicrobial peptides have been targeted toward specific pathogens as well. For example, *E. coli* Nissle 1917 (EcN) has been engineered to express three proteins, pyocin S5, the E7 lysis protein, and the antibiofilm protein DspB, upon detection of the *Pseudomonas* quorum-sensing molecule *N*-(3-oxododecanoyl)-$_L$-homoserine lactone. This probiotic delivery method was proven effective in both treating and preventing *P. aeruginosa* gut infections in mice.[38] More recently, the same detection and expression system was used to express PA2-GNU7, a specific antimicrobial peptide, to eradicate *P. aeruginosa*.[94] Moreover, EcN has been applied to express microcin H47 upon detection of tetrathionate, a molecule produced during gut infection that enhances the growth of *Salmonella*.[95] EcN has also been engineered to produce bile salt hydrolase Cbh upon detection of sialic acid, a biomarker of dysbiosis. Expression of this protein restored the disturbed bile acid metabolism, and, as a result, alleviated *Clostridioides* infection.[96,97] Apart from *E. coli*, *Lactococcus lactis* NZ9000 has also been engineered to produce three different bacteriocins (enterocin A, enterocin P and hiracin JM79) upon detection of the enterococcal sex-pheromone cCF10.[41] As an alternative to the administration of engineered microorganisms, native microorganisms of the microbiota may be engineered *in situ* to express proteins. This development is fueled by recent advances in the engineering of microorganisms previously thought to be genetically intractable.[98] An attractive method for this may be using phages as vectors for specifically delivering recombinant DNA.[99] For example, Meile et al. engineered a coliphage to deliver an *Enterococcus*-targeting lysin gene, forcing the phage-infected *E. coli* cells to express the lysin. They demonstrated that both *E. coli* and *Enterococcus faecalis* reduced upon phage infection.[100] This method can be conceived as a hybrid technology, combining both phage therapy and lysin treatment to modulate a microbiome and alleviate polymicrobial urinary tract infections in which both *E. coli* and *E. faecalis* are the etiological agents. For a comprehensive review comparing phage therapy and phage enzymes, the reader is referred to.[101]

Microbiome-friendliness of lysins

While specificity aims to narrow down the activity range of a lysin *in vitro*, care should be taken when drawing conclusions from *in vitro* specificity data and when extrapolating specificity to microbiome-friendliness. That is, such a specificity test cannot take the complexity of the *in vivo* environment into account. Therefore, microbiome-friendliness should be assessed by sequential *in vitro*, *ex vivo*, and *in vivo* experiments.[28] During such assessments, the composition of the microbiome can be evaluated by culture-based techniques, together with sequencing methods to account for uncultivable members of the microbiome. Moreover, the metabolic and functional capacity can be evaluated

using metatranscriptomic, metaproteomic, and/or metabolomic techniques. These research areas are rapidly evolving, possibly enabling a more in-depth evaluation of microbiome-friendliness in the future.[102,103]

Multiple *in vitro* models have been developed to examine intestinal microbial communities, as reviewed elsewhere.[104] For instance, fermentation models can be used to study microbiota activity and dynamics. These models typically consist of one (single-stage) or multiple (multi-stage) reactor vessels, in which specific (micro)-environments of the gastrointestinal tract are simulated. In the case of single-stage fermentation models, both batch[105] and continuous systems[106–109] are used. On the other hand, multistage fermentation models are always continuous systems and they further vary in other operational conditions, such as the number of reaction vessels.[110–112] These continuous models attempt to give a more realistic approximation of the conditions within the (gastro-)intestinal tract. This is done by, for example, taking longitudinal differences along the gastrointestinal tract (*i.e.* between the stomach, small intestine, proximal and distal colon)[113] and cross-sectional differences within the gastrointestinal tract (*i.e.* between lumen and mucosa)[114] into account. Depending on the research question and available resources, one or more *in vitro* models can be used or combined. In general, fermentation models can provide an idea of microbiome activity and compositional microbial changes, including the complex interactions that may occur at a mixed population level, while staying in a relatively controlled *in vitro* environment. However, they still do not incorporate the potential effects of host interactions. To address this limitation, microfluidic-based models have been developed that simulate the complex physiology and structure of the intestine in a miniature flow system. These *in vitro* models usually consist of multiple microchannels, representing microchambers, in which human intestinal Caco-2 cells or microbiota are grown, or through which medium is dispended.[115–119] Because *in vitro* models are in general less expensive, more stable, and robust, and have fewer ethical constraints compared to *in vivo* models, they are considered a good starting point to assess microbiome-friendliness. Still, they are unable to capture more complex reactions after treatment, and can merely give a first insight into potential interactions with the intestinal epithelium.[104] Therefore, additional experimental setups are also typically required to assess microbiome-friendliness.

In contrast to *in vitro* models, *ex vivo* models include functional tissues of animal origin, thereby including more of the cellular complexity found in an *in vivo* environment. The Ussing chamber,[120–122] the Everted sac model,[123] the InTESTine™ model,[124] biopsy-derived organoids,[125–127] and biopsy-derived microfluidic-based chips[127–129] are examples of *ex vivo* models for the intestinal tract. *Ex vivo* models allow the simulation of more complex functional environments, and thus assessment of treatment impact at physiologically relevant conditions. Nevertheless, these models are generally more expensive, less scalable and reproducible and enable a lower throughput compared to *in vitro* models.[130] Moreover, both *in vitro* and *ex vivo* models are not able to capture more complex, systemic interactions, such as neurological and immunogenic reactions. Yet, such effects are particularly interesting, as the gut and its microbiome are interconnected with host organs and the immune system. This, in turn, can affect treatment outcomes. For example, one study found that a bacteriophage cocktail targeting commensal *Klebsiella pneumoniae* alleviated symptoms in patients with inflammatory bowel disease by specifically eliminating bacteria-induced inflammation.[131] In contrast, another study found that a bacteriophage cocktail aggravated intestinal colitis in IBD patients by the intrinsic immunomodulatory effects of the bacteriophages.[132] Therefore, *ex vivo* models should be merely considered a second step toward assessing the impact of a treatment, before moving on to *in vivo* experiments.

Both animal models and human clinical trials are used to perform *in vivo* experiments. These studies are the most physiologically relevant because they consider both the impact of treatment on the microbiome and the crosstalk between the host and the microbiota. However, challenges to sample specific locations and ethical issues arise when performing experiments on animals or humans, apart from them being time- and cost-

intensive. Moreover, achieving a stable colonization of specific species pathogenic to humans in animals can be challenging.[133] In addition, the biological structure and organization of animals vary, complicating the extrapolation of the results of *in vivo* animal experiments to humans and impacting their relevance.[104] Still, *in vivo* experiments are crucial to assess the safety and efficacy of a compound.[134]

Although the specificity of lysins against a panel of bacteria is almost standardly examined, the follow-up assessment of microbiome-friendliness is usually not implemented. While *in vitro* and *ex vivo* models have already been performed to assess the microbiome-friendliness of lysins for the skin[49] and the vaginal microbiome,[51] up till now, no such studies have been done regarding the intestinal microbiome. However, the impact of other antibacterial proteins has been tested using *in vitro* and *ex vivo* models, for example for the bacteriocins nisin,[135] pediocin PA-1[136] and plantaricin NC8,[137] which shows that these models are amenable for assessing microbial impact of proteins as well.

Up till now, only a limited number of *in vivo* studies have been performed to evaluate the impact of a lysin on the intestinal microbiome. On the one hand, Harhala et al. demonstrated that no significant microbiome compositional changes occurred in mice after intraperitoneal injection of a pneumococcal lysin, either Pal or Cpl-1.[138] On the other hand, the composition of the microbiome of mice changed after intraperitoneal injection of an enterococcal lysin, LysEF-P10, albeit not significant. More specifically, a reduction in the relative abundance of the Gram-positive phyla Firmicutes and the Gram-negative phyla Proteobacteria and Deferribacteres was observed, as well as an increased relative abundance of the phyla Bacteroidetes and Verrumicrobiota.[139] In terms of targeting Gram-negatives, administration of a broad-spectrum lysin targeting Gram-negative bacteria, being either LysAm24, LysAp22, LysECD7, and LysSi3, induced some observable changes in microbiome composition, albeit not-significant, after intraperitoneal injections in mice. In general, the major quantitative changes were observed as a reduction in the Gram-negative phyla Proteobacteria and Bacteroidetes and an increased abundance of the Gram-positive phylum Firmicutes.[140] These apparent contrasting results show that each lysin has a different impact on the microbiome and thus should be evaluated individually, including at lower taxonomic levels, instead of assuming microbiome-friendliness based on the specific nature of a lysin. Moreover, minor differences between methods during the assessment could also contribute to the contrasting results. Therefore, standardized approaches should be implemented in the evaluation of microbiome-friendliness.

Delivery of proteins in the gut

Oral administration is the preferred route for microbiome modulation because of patient compliance, low cost, and the noninvasive character. However, this requires the protein to transit unharmed through the intestinal tract, a process complicated by the proteolytic action of digestive enzymes, which can degrade the enzymes, and a low pH in the stomach, which can irreversibly disrupt protein structure. To overcome these hurdles, multiple techniques and materials have been developed, including the administration of probiotics that recombinantly express the protein of interest, and formulation of the protein of interest using particle-based or macroscopic systems.[141]

Probiotic delivery

True probiotics are defined as viable and active microorganisms that confer a health benefit on the host when administered in sufficient amounts.[142] While the administration of probiotics in itself is considered a viable technique for microbiome modulation, probiotic strains can be engineered, *e.g.*, to recombinantly express phage-derived lysins, adding a specific antibacterial activity against pathogenic strains to their already beneficial probiotic effect.[143]

Phage-derived lysins have already been expressed in bacterial and yeast probiotic strains (Table 1). For example, the Gram-positive probiotic strains *Lactobacillus johnsonii* FI985[144] and *Lactococcus lactis* NZ9000[146,147] were already applied to produce and secrete phage-derived lysins. More specifically, *L. johnsonii* FI9785 produced CP25, a lysin with lytic activity against its target bacterium, *Clostridium perfringens*.[144] *L. lactis* NZ9000 was able to express

Table 1. Probiotic delivery methods to deliver phage lytic enzymes.

Expression host	Protein expressed	Target bacterium	Observation	References
Lactobacillus johnsonii FI9785	Endolysin CP25L of *Clostridium perfringens* prophage vB_CpeS-CP51	*Clostridium perfringens*	*L. johnsonii* FI9785 constitutively expressing and secreting active endolysin CP25L, which reduces *Clostridium perfringens* in turbidity reduction assay and breaks down autoclaved *Clostridium perfringens* cell walls.	[144,145]
Lactococcus lactis NZ9000	PlyE146	Gram-negative bacteria	*L. lactis* NZ9000 expressed PlyE146 with His-tag upon induction with nisin. Purified protein had bactericidal activity against *E. coli*, *Acinetobacter baumannii* and *P. aeruginosa.*	[146]
Lactococcus lactis NZ9000	Endolysin Endo88 and virion-associated peptidoglycan hydrolase VAH88 *S. aureus* bacteriophage 88	*S. aureus*	*L. lactis* NZ9000 expressed Endo88 and VAH88 with His-tag upon induction with nisin. Purified proteins had muralytic and growth inhibition effect on *Staphylococcus* strains.	[147]
Saccharomyces cerevisiae strain BY4727	LysA and LysA2 of respectively *Lactobacillus johnsonii* prophage Lj928 and *Lactobacillus casei* bacteriophage A2	*Limosilactobacillus fermentum*, *Levilactobacillus brevis*, *Lactobacillus mucosae*	Purified LysA and LysA2 expressed by *S. cerevisiae* showed bacteriolytic activity, although bacteriolytic activity was higher for the same amount of purified enzyme expressed by *E. coli*. Induced *S. cerevisiae* reduced the presence of *L. fermentum* in a fermentation setup.	[148]
Saccharomyces cerevisiae EBY100	LysKB317	*Limosilactobacillus fermentum*	*S. cerevisiae* cells displayed the lysin LysKB317. *S. cerevisiae* expressing LysKB317 reduced viable *L. fermentum* upon simultaneous incubation. *S. cerevisiae* expressing lysin LysKB317 reduced the abundance of viable *L. fermentum* in a corn mash fermentation setup.	[149]
Saccharomyces cerevisiae EBY100	Endolysin LysSA11 of staphylococcal phage SA11	*S. aureus*	*S. cerevisiae* cells displaying LysSA11 reduced the number of viable *S. aureus* cells.	[150]
Pichia pastoris X-33	Endolysin (Vplys60) of *Vibrio parahaemolyticus* bacteriophage qdv001	*Vibrio parahaemolyticus*	*Pichia pastoris* X-33 expressed Vplys60 with His-tag. Purified proteins were active, as determined by the turbidity reduction assay. Maximum yield was 340 ± 18 U/mg expression.	[151]
Pichia pastoris X-33	Endolysin LysP2 *Salmonella* phage YSP2	*Salmonella pullorum*	Pichia pastoris expressed LysP2 with an expression yield of 239 µg/mL. Purified protein was able to alleviate *Salmonella* infection in chickens.	[152]

PlyE146, a lysin targeting multiple Gram-negative bacteria, which showed bactericidal activity after protein purification.[146] Similarly, this same strain was able to produce two different lysins originating from *Staphylococcus* bacteriophage 88, Endo88, and VAH88.[147] The purified proteins possessed lytic and growth inhibitory activity against staphylococcal strains.[147] The ability of this strain to produce active lysins against both Gram-positive and Gram-negative bacteria could indicate its wide applicability as a delivery vehicle. However, problems with the toxicity of phage-derived endolysins toward bacterial expression strains can occur because the peptidoglycan of the bacterial delivery vehicle could possibly also be degraded by the protein. Yeast probiotic microorganisms could therefore be used to express phage-derived lysins, thereby circumventing these possible toxicity problems. While true yeast probiotic strains such as *Saccharomyces boulardii* have not yet been used for lysin secretion, work with *Saccharomyces cerevisiae* showed the potential to express different phage-derived lysins, which were shown to be active when displayed on the yeast surface.[149] Moreover, work with *Pichia pastoris* X-33, a *Pichia* strain with probiotic characteristics, demonstrated the capability of this strain to express a lysin, which was proven to be active after protein purification.[151,152]

Although probiotic strains have successfully produced phage-derived lysins, no studies have been reported on the delivery of these lysins to the intestinal tract by probiotic delivery vehicles. This could potentially be due to additional challenges encountered when engineering probiotics for the delivery of proteins to the gut. Specifically, the probiotic strain should not only produce the protein of interest in an *in vitro* pure culture but should be able to survive passage through the gastrointestinal tract, multiply within it, compete with resident bacteria for nutrients and space, and express the active protein of interest. Additionally, safety concerns about the application of bioengineered probiotics could hinder translation into pharmaceutical products. Therefore, multiple

biocontainment strategies have been developed to limit the dissemination into other environments and to regulate the temporal presence of the probiotic, including, *e.g.*, the engineering of kill switches and auxotrophic requirements into the probiotic strain.[153–155] Notwithstanding safety concerns, the use of engineered probiotics holds great promise for protein delivery to the gut. In this regard, a recent phase 1b/2a clinical study that used an engineered *Lactococcus lactis* strain expressing human proinsulin and IL-10 to treat the development of Type 1 diabetes is illustrative. The study showed that the administration of the bioengineered probiotic is safe and well tolerated by the patients. Moreover, metabolic variables stabilized or even improved upon administration.[156]

Protein formulation systems

Proteins can be formulated in specific ways to enhance their bioavailability by oral drug delivery. Indeed, multiple systems and materials are explored to circumvent their enzymatic degradation and pH-induced inactivation during gastrointestinal transit and to enhance protein absorption through the gastrointestinal barrier, as reviewed elsewhere,[141,157] and some systems could also be employed for delivery to the gut itself. In general, oral drug delivery systems of proteins are classified either according to their size into particle-based (having a size in the range of nano- to micrometer) and macroscopic systems (materials bigger than 0.1 mm),[141] or according to the material used for formulation into inorganic nanoparticles, polymer-, and lipid-based systems.[158] For example, proteins for oral delivery can be encapsulated into inorganic particles,[159] polymeric particles,[160] liposomal carriers made up of a lipid bilayer,[161] or by surfactant molecules into micelles.[162] In addition, proteins can be loaded onto polymeric nanofibers.[163] Besides those particle-based systems, macroscopic systems such as hydrogels are also being developed for the oral delivery of proteins.[164] By thoughtfully combining and choosing between techniques and materials, specific delivery properties can be obtained. For example, the site-specific release of proteins within the colon can be achieved by using materials that degrade or swell upon differences in pH[164,165] or interaction with the microbiota, microbial metabolites, or enzymes present in the gastrointestinal tract.[163]

Using one of the formulation techniques, the successful delivery of phage-derived lysins topically, systemically, pulmonary, and musculoskeletal, on catheters and implants has already been achieved, as reviewed elsewhere.[47,78,166–168] Moreover, encapsulation of complete phages has also been successfully used to deliver phages to the intestinal tract for microbiome modulation.[169,170] However, to our knowledge, these techniques have not yet been used to deliver lysins to the intestinal tract for microbiome modulation. At the same time, bacteriocins and other proteinaceous antimicrobial proteins, have been successfully formulated for oral administration to achieve microbiome modulation. For example, Carpena et al. developed hydrogel microcapsules, allowing pH-dependent release of colicin E9 and Ia bacteriocin, resulting in selective decolonization of the intestinal tract from *E. coli*.[168] Another research group developed a polymeric nanoparticle composed of milk protein for delivery of the pediocin from *Pediococcus pentosaceus* CRA51. These particles significantly reduced the number of *E. faecalis* MTCC 439 and *S. aureus* MTCC 740 cells adhering to HT-29 cells in an *in vitro* intestinal model.[171] Interestingly, a recent study found that both encapsulated and unencapsulated nisin, a broad-spectrum *Lactococcus lactis* bacteriocin, was able to modulate the gut microbiome of pigs.[172] This suggests that at least some proteins can transit intact through the intestinal tract to modulate the microbiome. Together, these encapsulation studies show the potential of particle-based and macroscopic systems to deliver proteins, such as phage-derived specific lysins, to the gut for microbiome modulation. However, more research is required to fully exploit their potential.

Conclusion

The importance of the microbiome on human health has gained increasing attention, and researchers have made increasing efforts to improve health by altering the function or composition of the microbiome during a process called microbiome engineering. In this review, opportunities and challenges to develop phage-derived lysins to modulate the gut microbiome by depleting specific species are identified. Phage lysins are enzymes that specifically degrade the bacterial cell wall and are considered to

be microbiome-friendly. To develop phage-derived lysins for microbiome modulation, several challenges are identified. First, although lysins are considered specific, their host range can vary from strain- to genus-level, or even broader, which can possibly result in off-target effects. Protein engineering and specific delivery vehicles can be implemented to overcome this hurdle. Second, lysin specificity does not always translate into microbiome-friendliness. While the meaning of microbiome-friendliness is equivocal, future research could give more information about microbiomes, sparking the debate to achieve a more clarifying definition. In any case, the impact of a treatment on a microbiome should be assessed using *in vitro*, *ex vivo*, and *in vivo* models. These models are already existing for the intestinal tract. Finally, delivery of a protein to the intestine can be challenging due to possible proteolysis and degradation within the gastrointestinal tract. The use of probiotic organisms and particle-based and macroscopic systems can be implemented to overcome this challenge.

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Data availability statement

No new data was produced in this work.

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