Review Article

The Challenge of Applications of Probiotics in Gastrointestinal Diseases

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Gastrointestinal disease is characterized by gastrointestinal dysfunction with dysbiosis of the microbiome. Probiotics may act as biological agents in treating gastrointestinal diseases through modifying gut microbiota. However, several challenges, including safety, stress resistance, postcolonization quantification, and evaluation models, may hinder the application of probiotics in gastrointestinal diseases. This review introduces the emerging methods for delivering probiotics as well as available materials. Furthermore, we elucidated bacteriocins and their role in helping probiotics obtain a competitive advantage over other strains and challenges of large-scale application. Bacteriocins produced by probiotics also showed promising efficacy in gastrointestinal diseases including the capacity of immune stimulation, intestinal barrier protection, and cytotoxicity against intestinal tumorigenesis. For the quantification of probiotics in complex microbiomes and evaluation methods of probiotic encapsulated delivery systems, recent fluorescent labeling technology and various in vitro and in vivo models were also reviewed. Given the widespread use of probiotic agents in the microecological therapy of gastrointestinal diseases, further understanding of the multiple challenges of probiotic application and the updated methods to improve the colonization and evaluation system of probiotics is of great significance for probiotics as live biotherapeutics.

1. Introduction

Although probiotics exhibit high potential as therapeutic agents in treating gastrointestinal diseases, the applications of probiotics are still facing challenges [1]. Firstly, probiotics must be safe for human consumption, without any transferable anti-biotic-resistant genes [2]. Therefore, engineered probiotics are usually not allowed to be used for treatment of diseases. Secondly, the minimum viable counts (~10^6 CFU/g) are required in order to be beneficial [3]. However, the most commonly used probiotics usually belong to Lactobacillus and Bifidobacterium, which are very susceptible to aerobic, and high-temperature environments, and the emerging next-generation probiotics demand more favorable conditions. Besides, probiotics must endure the stomach acid and bile during the gastrointestinal transition
Even though probiotics reach the colon alive, they may have to adhere to the mucus layer and colonize the colon in order to be effective. Therefore, design of probiotic formulations for targeted delivery to the intestines is quite challenging. Bacteriocins could act as a “colonizing peptide,” “killing peptide,” and “signal peptide” by promoting the colonization of the producing strain in the gut to gain a competitive advantage over other strains. Thirdly, in quantifying probiotics, many problems still need to be solved, such as on-site localization and dynamic monitoring. The existing feces examining methods cannot satisfy fast-developing gut microbiota studies [5]. Therefore, new tools are urgently needed, such as fluorescent labeling and imaging of gut microbes. Last but not least, establishment of more proper \textit{in vitro} and \textit{in vivo} models for evaluating the function of probiotics or probiotic encapsulated delivery systems is required. By solving these problems, probiotics may exhibit good prospects in the food and pharmaceutical fields in the future. Here, we discuss the potential therapeutic challenges of probiotic application and updated recent progress.

2. Targeted Delivery of Probiotics

With the ever-growing health needs of people, delivery of probiotics through dietary supplements, foods, and beverages has become increasingly popular. Such dietary supplements and nutritionally enhanced foods are appealing to different groups of people with high health care demand, such as women, infants, children, adolescents, the elderly, and those recovering from wounds or surgery [6]. The best-known functional foods with added probiotics include yogurt, cheese, ice cream, and other dairy products, while novel nondairy products emerged recently, and the market keeps expanding [7]. Moreover, novel snacks which have been incorporated with desired strain combinations include chocolate bars, cereal, juice, and chips [8].

Successful incorporation of probiotics into dietary supplements or foods requires careful experiments to find out optimum strain combination and formulation to deliver them. Challenges such as pH and water activity adjustment, temperature control, shelf-life evaluation, and sensory concerns need to be overcome before a perfect product can be achieved for consumers [9, 10]. Normally, low storage temperature and high-fat content are favorable conditions for the incorporation of probiotics, which make dairy products ideal candidates for probiotic-enhanced foods. Encapsulation and controlled release systems which are popular among pharmacists have been adopted to stabilize the probiotics in certain matrices of foods or beverages recently, resulting in more varieties of snacks containing healthy probiotics [11, 12].

Most recently, there has been an increased interest in targeted delivery of probiotics. Normally, such targeted delivery is aimed at getting the probiotics to the intestine so that better health benefits can be achieved, including enhancing gastrointestinal stability, reducing lactose intolerance, relieving diarrhea, boosting immunity, and lowering cholesterol [13–15].

The formulation of probiotics for targeted delivery to the intestines can be quite challenging in order to resist the strong acidic gastrointestinal environment. Exposure to gastric acid can be devastating to unprotected probiotics, and this is why coating layers and encapsulation techniques must be applied to protect the probiotics from such strong acidic environment until they are delivered to the targeted place [16]. The most commonly used encapsulation approaches are pH-sensitive and bacteria sensitive coating layers, which can release the coated probiotics in the intestine in response to specific pH conditions or certain bacteria colonies [17] (Figure 1). Other critical considerations include utilization of natural and economical coating materials; increasing the adhesion of the outer surface of the coating to epithelial cells of the intestines; enhancing the bioavailability, bile salt hydrolase activity, stability of the probiotics, antagonistic activity, and efficacy; and targeting capacity of the delivery and safety concerns related [10].

Various wall materials have been investigated to achieve the goal of targeted delivery of different probiotics, and the most widely used include dietary fibers, proteins, and natural polysaccharides [18]. Synthetic materials with good biocompatibility have been used as well. To achieve better efficacy, combinations of different wall materials have also been attempted, and emulsifiers are added as match makers. More importantly, the stability of the probiotics itself has to be taken into account, especially during the drying step of food processing [19]. Here, the drying techniques usually include freeze drying, spray drying, extrusion, refraction window drying, electrospaying, electrospinning, and emulsifying. The cost and the temperature of each drying process are important factors to choose a proper processing approach [6]. Additionally, advanced techniques such as microfluidics, genetic engineering, and 3D printing have also been employed to achieve enhanced encapsulation efficiency recently [20].

3. Bacteriocins: Advantages and Challenges

Bacteriocins are antimicrobial peptides synthesized in the ribosome [21], and many probiotic strains were found with a capacity of producing bacteriocins, like the strain \textit{Lactococcus lactis} which can produce nisin [22, 23]. Bacteriocins are classified into four classes according to their molecular weight, chemical structure, thermal stability, and modification [24–26]. It is generally believed that bacteriocins kill the targeted cells by destroying the integrity of cell membranes [27], cracking bacterial DNA, interacting with intracellular enzymes, and interrupting bacterial protein synthesis [28, 29]. Class I bacteriocins are small posttranslationally modified ribosomal peptides, which is the class nisin belongs to [30] (Table 1). Class II bacteriocins are small thermostable peptides (5–10kDa) and nonmodified proteins (such as pediocin PA-1 and plantaricin EF) [31, 32]. Class III bacteriocins are macromolecular heat-sensitive proteins (>30kDa) (such as \textit{Lactococcus} 972) [33]. Class IV bacteriocins consist of large complexes with carbohydrates or lipid moieties (such as \textit{Lactocin} 27) [34]. In addition to the above
Drissi et al. reported that the firmicutes cyclic bacteriocins ‘killing peptide,’ ‘colonizing peptide,’ and ‘signal peptide’ by promoting the colonization of the producing strain in the gut to gain a competitive advantage over other strains [23], targeting colonization of vancomycin-resistant enterococci (VRE) in vivo [38]. Garvicin ML, produced by L. garvieae DCC43, was able to improve host health by modifying intestinal microbiota, which dominate the human gut microbiota, can produce the largest number of bacteriocin (about 70.79%) [36]. Among them, the vast majority bacteria encoding bacteriocins belong to lactic acid bacteria (LAB), indicating that bacteriocins from LAB play a crucial role in maintaining the balance of gut microflora. Bacteriocins could act as a “colonizing peptide,” “killing peptide,” and “signal peptide” by promoting the colonization of the producing strain in the gut to gain a competitive advantage over other strains [23], targeting undesirable pathogens without negatively affecting beneficial flora [37], and participating in quorum sensing with other bacteria or to signaling cells in the host immune system, respectively. Pediocin PA-1, produced by Pediococcus acidilactici UL5, was reported to reduce the intestinal colonization of vancomycin-resistant enterococci (VRE) in vivo [38]. Garvicin ML, produced by L. garvieae DCC43, was able to improve host health by modifying intestinal microbiota, including potentially problematic bacteria inhibition, LAB proportion increase, and triglyceride level decrease [23].

Bacteriocins are involved in immune regulation and intestinal epithelial barrier protection, thus controlling diseases such as obesity (Table 2) [39, 40]. Nisin and CBP22 were found to protect the host from pathogens’ infection by enhancing the intestinal immunity [41, 42]. Nisin could also affect neutrophils and induce the formation of neutrophil extracellular traps in vitro in a dose-dependent manner [41, 43]. Yin et al. reported that plantaricin P1053 can reduce the proliferation of intestinal cancer cells while enhancing the vitality of healthy cells [47]. In addition to its anti-tumor activity in vitro, bacteriocins also show good efficacy in vivo studies on tumor inhibition [48, 49]. Reuterin is a class II bacteriocin isolated from L. reuteri. Bell et al. found that tumor volume of mice treated with reuterin was significantly reduced when the nude mouse model was implanted by HCT116 and SW480 colon cancer xenograft [45]. Goyert et al. found that reuterin can inhibit the growth of colorectal cancer by altering the redox balance [50].

Bacteriocins have proved to be an important weapon in maintaining microecological balance and gut health [51]. However, there are certain challenges in bringing bacteriocins into large-scale application. Firstly, bacteriocins obtained by traditional separation methods such as genetic engineering to construct recombinant cells have low yields and take a long time, while chemical synthesis methods are expensive and not suitable for large-scale production [52]. Furthermore, another issue is the instability of bacteriocins under low/high pH and pepsin influenced conditions [51]. Meanwhile, the degree of expression of bacteriocins under harsh gastrointestinal conditions has not been elaborated, and further research is required to establish a method to improve the stability and efficacy of bacteriocin as biologics.

### 4. Quantification of Probiotics: Fluorescence Labeling

At present, the identification and quantification of most gastrointestinal microbiomes are highly dependent on the high-throughput DNA sequencing of fecal microflora [53]. The gut microbiota is composed in a certain proportion to form a stable ecosystem, where bacteria cooperate and restrain each other. It is controversial whether the fecal microbiome can represent the whole composition of the colonized bacteria in the gut [54, 55]. Simultaneously, many problems still need to be solved in quantifying probiotics, such as on-site localization and dynamic monitoring. Many foreign bacteria are excreted with the feces as they cannot colonize in the intestinal tract. The composition of bacteria in feces exceeds that of colonized gut microbiota. The existing feces examining methods cannot satisfy rapidly developing gut microbiota studies. Therefore, new tools are urgently needed to detect microbial communities and their presence in the digestive tract. Gut microbes’ fluorescent labeling and imaging could be an appropriate method to resolve this problem.

Researchers have recently been devoted to developing convenient bacteria fluorescent imaging methodologies. Transferring fluorescent protein genes into bacteria genes by regulating the levels of TNF-α and IL-6 [44], and it also protects the epithelial barrier by increasing ZO-1 expression in the ileum in obese mice Heeney et al. [31].

Bacteriocins also appear to have selective cytotoxicity against colorectal cancer cells in comparison to normal cells [45], enabling inhibition of intestinal tumorigenesis. In vitro assays revealed that different concentration of nisin inhibited the proliferation of SW48, HT29, and Caco-2 cells to different degrees [46]. De Giani et al. reported that plantaricin P1053 can reduce the proliferation of intestinal cancer cells while enhancing the vitality of healthy cells [47]. In addition to its anti-tumor activity in vitro, bacteriocins also show good efficacies in vivo studies on tumor inhibition [48, 49]. Reuterin is a class II bacteriocin isolated from L. reuteri. Bell et al. found that tumor volume of mice treated with reuterin was significantly reduced when the nude mouse model was implanted by HCT116 and SW480 colon cancer xenograft [45]. Goyert et al. found that reuterin can inhibit the growth of colorectal cancer by altering the redox balance [50].

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was initially attempted [56, 57]. However, most of the gut microbiota is hard to isolate and culture under artificial conditions, making the gene transfer challenging [58]. Thus, in vitro testing tools based on bacterial nucleic acid sequencing, such as fluorescence in situ hybridization (FISH), were adopted [59]. It is unfortunate that FISH can only label dead bacteria. Therefore, many attempts have been made to label bacteria in vivo.

Since some antibiotics can specifically bind to the bacterial outer membrane, antibiotics are conjugated with fluorescent dyes to form bacterial targeting probes, which can selectively label bacteria in complex samples [60–62]. The combination of lipopolysaccharide-targeted polymyxin B and Cy3 dye can form a Gram-negative bacterial-specific probe [61]. The peptidoglycan-targeted vancomycin Cy3 conjugation probe could label Gram-positive bacteria after incubation with intestinal flora [62]. The most significant concern of those probes is the toxicity. Even low concentrations of antibiotic-based imaging probes could damage bacteria, resulting in drug resistance and host-microbiota disorder [63, 64].

Consequently, the metabolic labeling strategy is proposed to solve this problem. Metabolic-based mimic probes such as unnatural precursors or substrates could tag bacteria during proliferation or energy harvesting. And it has been applied to track gut microbiota colonization and spatial distribution [65]. The near-infrared sulfide quantum dots (PbS QD) coated with ribonuclease A (RNase-A) could be assembled with bacterial surface proteins [66]. The filamentous temperature-sensitive protein (FtsZ) inhibitor oxazolobenazamide is attached to a fluorophore that can mark Staphylococcus aureus, Enterococcus faecalis, Streptococcus pneumoniae, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa [67]. But this strategy would fail if the bacterial metabolic pathways changed, which is likely to happen in gut microbiota.

Due to negative surface charges, different metal cation sterilants are used [68]. Researchers also utilize the contrast reagent labeled metal cations for bacteria imaging. The zinc(II)-coordinated compound is combined with the near-infrared fluorescence group, and the probe has high specificity for wounds infected by Staphylococcus aureus [69]. Combining antimicrobial peptide G3KL with fluorescent dyes can target Gram-negative bacteria and accumulate on the cell membrane [70]. AIE material TBP-1 could target the cell membrane of methicillin-resistant Staphylococcus aureus with no drug resistance [71]. These probes have a simple chemical structures which are easy to synthesize, but they still face the same safety concern as antibiotic-based probes [72]. Positive charged dye MitoTracker Red is adopted in bacteria fluorescent imaging [73]. However, the cost is expensive due to the large amount needed for in vivo labeling by intragastric administration. On the other hand, some cation probes like cationic peptides could easily penetrate the mammalian cells, resulting in high background noise [74, 75].

In summary, despite deficiencies and shortcomings, fluorescence imaging technology is highly valued in probiotic quantification. The advantages of high sensitivity, accurate resolution, and low cost make it widely used in bacterial quantification and imaging. Better fluorescent probes and labeling strategies are eagerly needed to solve all the above concerns.

5. In Vitro and In Vivo Models

5.1. Bacterial Growth Inhibition Models. Escherichia coli, Salmonella, Listeria, Clostridium difficile, Helicobacter pylori, and Candida albicans are common pathogens that cause
gastrointestinal diseases. A large number of studies have used the traditional in vitro determination of the growth inhibition of probiotics on pathogenic bacteria to screen and assess the potential efficacy of probiotics (Figure 2). Abishad et al. investigated the effects of Lactobacillus acidophilus on a multi-drug-resistant enteroaggregative Escherichia coli (MDR-EAEC) strain, and they proved potential antibiotic and antibiofilm activity of green synthesized silver nanoparticles against MDR-EAEC strains with antioxidant properties [76]. Ruiz et al. used the symbiotic combination of Bifidobacterium longum subsp. infantis CECT7210 and oligosaccharides to examine the antimicrobial activity against Escherichia coli, Cronobacter sakazakii, Listeria monocytogenes, and Clostridium difficile in coculture experiments, and they found that the new symbiotic may be an effective supplement for infant health [77]. Cizeikiene and Jagelaviciute evaluated the antibacterial activities of twelve pathogenic strains belonging to Staphylococcus aureus, Escherichia coli, Staphylococcus chromogenes, and Staphylococcus hyicus species by performing agar diffusion assay and broth inhibition assay methods. The results demonstrated that Lactobacillus acidophilus DSM 20079, Bifidobacterium pseudolongum DSM 20099, and Bifidobacterium animalis DSM 20105 can serve as probiotic candidates [78].

Except for detecting the growth of pathogens, pathogenic genes or genes related to host infection were also utilized for screening probiotics. Wei et al. found that Bifidobacterium longum JDM301 not only played important roles in the growth inhibition against C. difficile but also directly promoted the degradation of clostridial toxin [79]. Ghadimi et al. evaluated the probiotic effects of Bifidobacterium animalis R101-8 by detecting the expression levels of key lipid metabolism genes, inflammation-related cytokines, and biomarkers, and they demonstrated that B. animalis R101-8 can improve biomarkers of metainflammation through the molecular/signaling mechanisms triggered by proinflammatory bacteria and lipids [80]. Additionally, with the rapid development of big data science and bioinformatics, some silicon models have also been developed for probiotic function evaluation. Mathematical models and genome scale metabolic models have been used for predicting and evaluating some bacterial probiotic functions [81, 82].

5.2. In Vitro Intestinal Microbiota Simulation Models. A more recent batch fermentation method simulating the distal colon could potentially be used for studying probiotic pathogen interactions. Models like SHIME, SIMGI, TIM-2, ECSTM, EnteroMix, and PolyFermS have been built for the human intestinal microbiota simulation [83]. Although most of these models were used for investigating the interactions between dietary functional factors or drugs and intestinal microbiota, the effect of probiotics, prebiotics, and symbiotics on the gut microbiota has been investigated by using these models. Duque et al. found that the probiotic, prebiotic, and symbiotic treatments resulted in a positive modulation of the gut microbiota and metabolic activity of children with autism spectrum disorder by using SHIME [84]. Marzorati et al. investigated the effects of MegaSporeBiotic™ (an oral, spore-based probiotic comprised of five Bacillus spp.) on gut microbiota activity and community composition using the SHIME, and they found that during treatment, Akkermansia muciniphila, Bifidobacteria spp., and Firmicutes increased while Lactobacillus spp. and Bacteroidetes decreased [85]. Due to the limited studies, the usability and feasibility of in vitro intestinal microbiota simulation models need to be further investigated.

5.3. Cell and Organoid Models. There are different models available that mimic the human intestinal epithelium and are thus available for studying probiotic and pathogen interactions in the gastrointestinal tract. Standard 2D models are comprised of culture plates as well as Transwell inserts, and newer 3D models like organoids, enteroids, and organ-on-a-chip have been built to assess probiotic-pathogen interactions [86]. Chen et al. found that the inhibition of H. pylori adhesion and the invasion of gastric epithelial cells and interleukin-8 production were significantly decreased by treatment with the Lactobacillus strains by using an in vitro cell-based model [87]. Wei et al. proved that Bifidobacterium longum JDM301 partially relieved damage to tissues caused by C. difficile and also decreased the number of C. difficile and toxin levels by using in vitro cell models [79]. Engevik et al. assessed the role of Lactobacillus reuteri in modulating the host immune system in an organoid-dendritic coculture and demonstrated that both L. reuteri secreted factors and its bacterial components are able to promote dendritic cell maturation [88]. In addition, some ex vivo models, like the Microbiota-human Intestine on chip (MihI-oC) [89], the Ussing chamber [90], and the human intestinal in vitro organ culture (IVOC) model [91], also have been developed. Efforts are under way to develop broader systems that connect multiple organotypic models to ultimately converge into a “body-on-a-chip” [92].

5.4. Animal Models. Animal models provide very controlled environments and enable the use of germ-free animals for studying the interactions between host and microbe as well as potential pathogens. In addition, animal models provide the possibility to collect samples from different parts of the gastrointestinal tract that is not possible within clinical trials. Although mice and rats are most frequently used, Caenorhabditis elegans, honey bees, Ciona robusta, fruit flies, and greater wax moths also have been developed for assessing probiotic-pathogen interactions [86]. Chen et al. indicated that colonization of H. pylori and induced stomach inflammation were alleviated by Lactobacillus strains [87]. Scafaro et al. used G. mellonella to evaluate the antibacterial activity of L. rhamnosus GG and Clostridium butyricum Miyairi against three enteric pathogens causing infections, and these results suggested that G. mellonella larvae are a potentially useful in vivo model, which can complement in vitro assays to prescreen candidate probiotics [93]. Although genome editing technology has made rapid progress and many genome editing rats and mice have been established, there are still few cases of genome editing animals used in probiotic-related research. Hence, more studies on animal models are needed.
5.5. Human Clinical Experiments. Regardless of the fact that in vitro models and animal experiments have the advantages of simple operation, controllable experimental factors, and low research cost, reliable evidence of the impact of probiotics on human health still largely depends on human clinical experiments. Numerous studies have also further promoted the development and sales of probiotic products through human clinical trials. For example, *E. coli* includes a variety of strains, most of which are regarded as opportunistic pathogens. Human clinical trials are one of the important reasons why *Escherichia coli* Nissle 1917 is recognized as a probiotic and widely accepted and used [94]. Dronkers et al. performed the global analysis of clinical trials with probiotics, and they found that *L. rhamnosus* GG and *Bifidobacterium animalis* ssp. *lactis* BB12 are the most widely studied probiotic strains [95]. Although there exist several clinical trials to investigate the benefits of probiotics in gastrointestinal diseases, the results can be inconsistent and sometimes contrary, which may cause by many factors, including trail design, group size, group characteristics, and dosage. Hence, the design of human clinical experiments should consider probiotics, host population, and study design carefully.

6. Conclusions

Although probiotics can serve as therapeutic agents in treating gastrointestinal diseases, there are still several challenges, which may limit the effective applications. Understanding the existing challenges will make better use of probiotics in gastrointestinal diseases. Importantly, if probiotics cannot be delivered to the targeted place, the anticipated effect will not be achieved. Bacteriocins and bacteriocin-producing probiotics can maintain microecological balance and gut health, whereas troubles such as large-scale production and instability under the certain environments limit their further application. For probiotic quantification, fluorescence imaging technology has been concerned, and better fluorescent probes and labeling strategies are required to solve the existing drawbacks. Up to now, several in vitro and in vivo models have been developed to assess the potential efficacy of probiotic, including bacterial growth inhibition models, in vitro intestinal microbiota simulation models, cell and organoid models, animal models, and human clinical experiments. Nevertheless, the development of these models is still in the initial stage, and more efficient and proper models need to be established.

**Conflicts of Interest**

The authors declare no competing interests.

**Authors’ Contributions**

Hang Xiao and William Wolfe developed the idea, and William Wolfe drafted the manuscript. Ze Xiang, Xi Yu, and Ping Li helped with the table and figures. Hao Chen, Mingfei Yao, Yiqiu Fei, and Yilun Huang revised the manuscript. Yeshi Yin developed the idea and drafted the outline. Hang Xiao organized, reviewed and finalized the manuscript. All authors contributed to the manuscript and approved for publication.

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