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PII: S0168-3659(20)30527-7

DOI: https://doi.org/10.1016/j.jconrel.2020.09.011

Reference: COREL 10521

To appear in: *Journal of Controlled Release*

Received date: 10 July 2020

Accepted date: 5 September 2020

Please cite this article as: S. Li, W. Jiang, C. Zheng, et al., Oral delivery of bacteria: Basic principles and biomedical applications, *Journal of Controlled Release* (2019), https://doi.org/10.1016/j.jconrel.2020.09.011

Oral delivery of bacteria: basic principles and biomedical applications

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Abstract

Bacterial therapy, which presents a smart platform for delivering and producing therapeutic agents, as monotherapy or in combination with other therapeutic modes, has provided a breakthrough for the treatment of a range of diseases. The integration of synthetic biology technology with bacteria enables their characteristics like chemotaxis and biomolecule secretion to outperform conventional diagnostics and therapeutics, thereby facilitating their clinical applications in a range of diseases. Compared to injection-administered bacteria, orally-delivered bacteria improve patient compliance while avoiding the risk of systemic infections. However, oral administration of microbes always leads to a substantial loss of viability due to the highly acidic environment in the stomach and bile salt in the intestine. Thus, the formulation of these bacteria into microcapsules using appropriate biomaterials is a promising approach for reducing cell death during gastrointestinal passage and controlling the release of these therapeutic cells across the intestinal tract. In this review, we reveal the basic principles of oral bacterial delivery, from internal genetic engineering approaches to external encapsulation and modification, and summarize the most recent biomedical applications. Finally, we discuss future trends in oral bacterial therapy as well as current challenges that need to be resolved to advance their clinical applications.

Keywords: oral bacterial delivery, biomaterial, encapsulation technology, genetic engineering, biomedical application

1. Introduction

Bacteria, which exist extensively in our human body, such as the cutis, nasopharynx, oral cavity, respiratory tract, gastrointestinal tract and female reproductive tract, have intensive involvements in human health, including metabolism, immunity and the gut-brain axis [1-7]. Taking advantage of the close connections between bacteria and the host, various bacteria have been investigated in the application of biomedicines for disease treatment, such as cancer [8], diabetes [9], gastrointestinal diseases [10], and obesity [11]. Some bacteria, including *Clostridia*, *Bifidobacteria* and *Salmonella*, are able to colonize the hypoxic area of the tumor and destroy the tumor cells [3, 12]. Other species of intestinal bacteria can induce anti-tumor immunity and regulate responses to immune checkpoint blockade [13, 14]. Moreover, numerous bacterial strains have been considered as drug delivery vectors, owing to their natural ability to accumulate in specific niches [15, 16]. Recently, in order to design bacteria as "smart" therapeutics and diagnostics for further clinical applications, genetic engineering and synthetic biology techniques have been used to precisely modify the bacterial cells and control their behaviors, such as environmental sensing, disease-site targeting and therapeutic release [17, 18]. Based on these strategies, the utilization of bacteria in disease management has outperformed conventional diagnostics and therapeutics, facilitating their translation into biomedical products for clinical application.

During the clinical application of bacterial therapy, oral delivery of bacterial products is the most convenient method of administration due to improved patient compliance [19]. However, the viability and activity of bacteria will significantly decrease after oral administration because of the low pH environment in the stomach and high bile salt condition in the intestine, which reduces their therapeutic effects *in vivo* [20-22]. Currently, encapsulation of the bacteria in a protective matrix is still considered as one of the most effective methods for reducing losses in viability after oral administration [21]. Such encapsulation could minimize bacterial death and maximize therapeutic effectiveness after

oral delivery [23]. The physical and chemical properties of the encapsulation materials are the major factors affecting bacteria survival and release rate at the target site. To date, numerous biomaterials, including polysaccharides (*e.g.*, alginate, pectin, cellulose, and chitosan), proteins (*e.g.*, whey protein, casein, and milk protein), and liposomes, have been used or have shown the potential to be used for encapsulation of bacteria [21].

Besides the inherent properties of the encapsulating materials, the manners, in which bacteria are enveloped, also play a crucial role in the protection of bacteria from the harsh gastrointestinal environment. With the successful application of oral administration of probiotics in the food industry, researchers have learned from similar methods of bacterial encapsulation and modification to expand their biomedical applications, especially for disease treatment [24, 25]. The utilization of appropriate encapsulation technology can protect bacteria during oral delivery, allowing them to achieve targeting abilities and desirable therapeutic effects.

Thus, by carefully designing smart therapeutic bacteria using synthetic biology technology and rationally selecting appropriate material and technique for bacterial encapsulation, oral delivery of novel bacteria products could be applied to a large scope of biomedicine fields. In this review, we summarize the basic design principles of oral delivery of bacteria, from internal genetic engineering to external encapsulation and modification, highlight the recent representative progress of orally-administrated bacterial therapy for biomedical applications, and discuss current challenges as well as future trends in this field.

2. Bacteria Used for Disease Management

The invention of the first microscope by Antony van Leeuwenhoek revealed a new world of microbes [26]. About 200 years later, Louis Pasteur and Robert Koch revealed the interactions between microorganisms and other creatures, indicating the establishment of microbiology [27]. A deeper understanding of the interaction mechanism between

microorganisms and the human body advanced their therapeutic role in disease management, which facilitated the development of the microorganism-based pharmaceutical and medicinal technology. As the main microorganism-based therapeutics, bacteria have attracted more and more attention for disease treatment in recent decades [28]. In modern microbiology, bacterial therapy has integrated with genome editing technology, such as clustered regularly interspaced short palindromic repeats (CRISPR)/associated protein 9 (CRISPR/Cas9), and synthetic biology to construct "smarter" therapeutics for a broad scope of clinical applications [29]. The recent progress of bacterial therapy is summarized in **Fig. 1**.

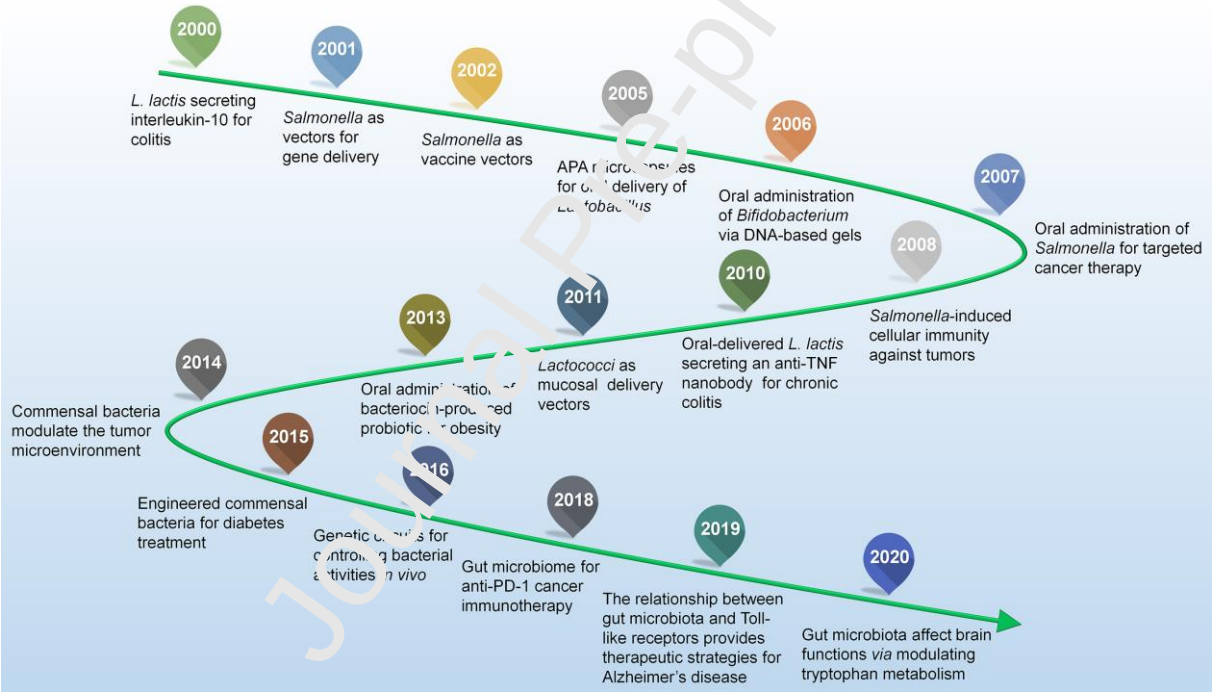

Fig. 1. Recent progress of bacterial therapy [30-42].

Currently, therapeutic bacteria that are commonly utilized for clinical application of disease treatment include *Escherichia coli* (*E. coli*) [43], *Salmonella* [44, 45], *Bacteroides* [46, 47], *Lactobacillus* [40, 48], *Bifidobacterium* [49], *Streptococcus* [50, 51], and *Listeria* [52, 53]. The specific application of bacterial therapies highly depends on their species and strain as well as their effector functions, such as the colonization of disease sites and the production

of therapeutics [17]. The clinical studies of bacteria for disease management are listed in **Table 1**, and **Fig. 2** consists of a brief diagram of these microbes. The unique properties of these bacteria that determine their potential use for disease treatment will be discussed below.

Table 1. The clinical trials of bacterial therapies [Data from ClinicalTrials.gov, accessed on 1 June 2020].

Trial No.	Type of Bacteria	Indication	Status	Company/Institution
NCT00004988	*S. typhimurium* VNP20009	Neoplasm metastasis	Phase I	National Cancer Institute (NCI)
NCT01924689	*Clostridium novyi*-NT spores	Solid tumor malignancies	Phase I	BioMed Valley Discoveries, Inc
NCT00936572	Probiotics (La1, BB536)	Colorectal cancer	Phase I	University of Milano Bicocca
NCT02625857	*Listeria*	Prostatic neoplasms	Phase I	Janssen Research & Development, LLC
NCT03420443	Gut bacteria	Rectal cancer/ radiotherapy	Not applicable	Region Skane
NCT00585845	*Listeria* CRS-207	Malignant epithelial mesothelioma	Phase I	University of Pennsylvania Abramson Family Cancer Research Center
NCT02966457	*E. coli*	Hematological infection	Phase IV	Belarusian State Medical University
NCT03032354	*L. rhamnosus* GG and *B. lactis* BB12	Type 1 diabetes	Phase IV	Medical University of Warsaw
NCT01836796	*Lactobacillus* DSM17938	Type 2 diabetes	Not applicable	Sahlgrenska University Hospital Gothenburg
NCT01130207	Gut bacteria	Obesity	Completed	Upstate Medical University
NCT02496390	FMT	Non-alcoholic fatty liver disease (NAFLD)	Phase I/II	Lawson Health Research Institute
NCT02426567	Gut bacteria	Crohn's disease	Not applicable	University of Glasgow
NCT00510978	*Bifidobacterium*/ *Lactobacillus*	Ulcerative colitis/Crohn's disease	Phase II/III	Cork University Hospital
NCT01847170	FMT	Crohn's disease	Phase I	Beth Israel Deaconess Medical Center
NCT00587041	Probiotic preparations (Agri-King Synbiotic and Oxadrop)	Nephrolithiasis/Crohn's disease	Phase I/II	Mayo Clinic
NCT02108821	FMT	Inflammatory bowel diseases	Phase I	Children's Mercy Hospital
NCT01993524	*Lactic acid bacteria*	Bacterial vaginosis/vaginitis	Not applicable	IBSS Biomed S.A.

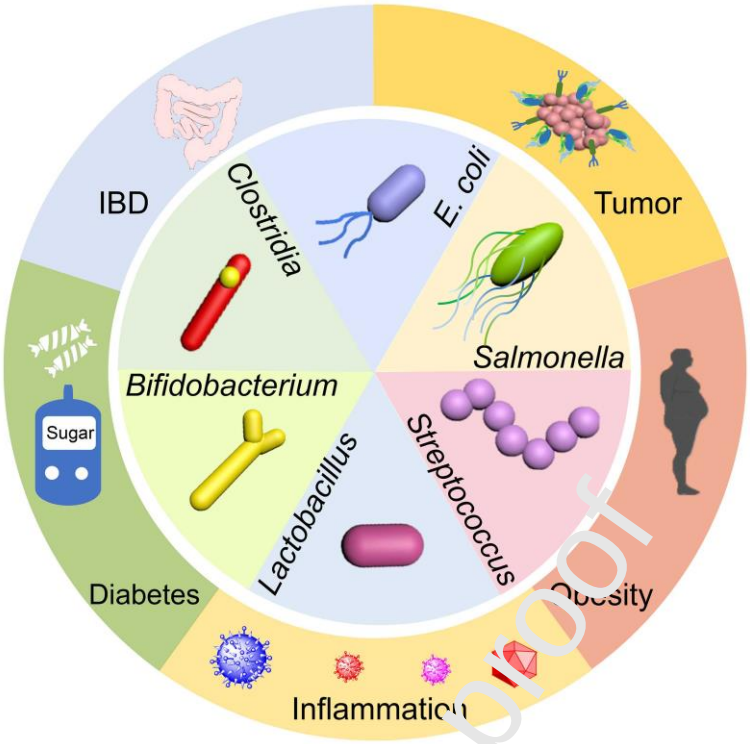

Fig. 2. An overview of commonly used bacteria for disease treatment. A variety of bacteria, including *E. coli*, *Salmonella*, *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, and *Clostridia*, have been widely used for managing various diseases, such as cancer, inflammatory bowel disease (IBD), inflammation, diabetes, and obesity.

2.1. *Escherichia coli*

E. coli, a Gram-negative facultative anaerobe, is one of the best-characterized model microorganisms, which have been extensively used for genetics, molecular biology, biochemistry and synthetic biology research [43]. As an inhabitant of the intestines and feces of warm-blooded animals and reptiles, this commensal strain does not produce virulence factors, so it is unable to induce damage to the surface of the intestinal epithelium [43]. Meanwhile, *E. coli* could stimulate the production of survival factors to support intestinal survival and modulate the mucosal inflammatory response by regulating the levels of proinflammatory cytokines. For these reasons, *E. coli* has been employed in several clinical trials for the treatment of intestinal infectious disorders and inflammatory bowel diseases [54,

55]. Moreover, *E. coli* strains are spontaneously capable of homing to tumor sites after systemically or local administration due to its anaerobic properties, making them ideal vectors for delivering or producing therapeutics for tumor imaging, diagnosis and treatment [56, 57]. For example, Yu *et al.* utilized the *luxCDABE*-transferred *E. coli* to visualize the localization of tumors and metastases in live animals because the light-emitting *E. coli* could localize, survive and replicate in solid tumors and metastases [58]. Terakawa and coworkers proposed a tumor-targeting system guided by anaerobic *E. coli*, which were genetically encoded to express the photosensitizer KillerRed for photodynamic treatment of tumor [59]. After subcutaneous injection of KillerRed-expressing *E. coli* into mice bearing with CNE2 cell xenograft tumor, the bacteria proliferated rapidly in the tumors because of their anaerobic properties. With the irradiation with an orange light (λ = 540-580 nm), the tumors became necrotic and were eliminated in a few days. Furthermore, Danino *et al.* used *E. coli* Nissle 1917 strains to design an diagnostic *via* oral administration, which could noninvasively indicate the presence of liver metastasis by generating easily determinable signals in the urine [60]. Owing to their anaerobic properties, these bacteria selectively colonized liver metastases to highly express a lacZ reporter. The expressed lacZ reporter could cleave a zymolyte to generate a signal molecule which could be easily examined in the urine. Despite the massive research about the treatment of gastrointestinal disorders and tumors using *E. coli*, more extensive studies are required to confirm these potentials in clinical trials.

2.2. *Salmonella*

Salmonella , a Gram-negative facultative anaerobe, is another widely studied bacterium [61, 62]. Due to their growth rate differences under aerobic and anaerobic conditions, *Salmonella typhimurium* (*S. typhimurium*) are able to colonize small metastatic and larger tumors, thereby inhibiting their growth [44, 63, 64]. Therefore, these strains are always employed as an anti-tumor agent for the treatment of various tumors [45]. However, the

systemic administration of *S. typhimurium* will lead to the pathogenesis in normal tissues and inducement of tumor necrosis factor α (TNFα)-mediated septic shock, limiting their further clinical translation. To overcome these limitations, these strains are always attenuated by auxotrophic mutations or genetically modified with lipid A-related mutations to lower their *in vivo* toxicity while retaining tumor-targeting and anti-tumor activity for the safe use in humans [44, 65]. Furthermore, these tumor-homing bacteria have also been utilized as gene vectors to deliver genes encoding angiogenic inhibitors [38, 44, 66-68], prodrug-converting enzymes [69], or cytokines [70-74], aiming to improve their oncolytic effects. Shiau's group has exploited *Salmonella choleraesuis* (*S. choleraesuis*) packaging an endostatin-encoding vector as a tumor-targeting therapeutic [66]. With the synergetic effects of tumor elimination and anti-angiogenesis, endostatin-expressed *S. choleraesuis* showed therapeutic potential for the treatment of solid tumors [67].

2.3. *Bacteroides*

Bacteroides groups, as the obligate anaerobic Gram-negative rods residing in the gastrointestinal tract, are generally considered to be the predominant genus of bacteria in the human colon [46, 47]. Although *Bacteroides* have been identified as the primary causes of severe infections [47, 75], recent studies have also revealed their close interactions with the immune system, appearing to determine the efficacy of checkpoint inhibitors [12]. Gajewski and co-workers noticed that the tumor growth of mice obtained from The Jackson Laboratory (JAX) or Taconic vendors with the same genetic background (C57BL/6) was varied, firmly dependent on their distinct microbial compositions [76]. Transplantation of fecal microbiota from JAX donors to Taconic receivers resulted in enhanced anti-tumor efficacy of PD-L1 antibody treatment. Thereafter, *Bifidobacterium* was identified by the authors as the essential role to mediate the enhancement of anti-PD-L1 efficacy by activating dendritic cells to stimulate the immune response of $CD8^+$ T cells for tumor elimination. A similar study by

Vetizou *et al.* also revealed a crucial role of *Bacteroidales* in the immunostimulatory effect of cytotoxic T lymphocyte antigen 4 (CTLA-4) blockade therapy [77]. Tumors bearing on antibiotic-treated or sterile mice exhibited no response to anti-CTLA-4 therapy. However, this deficiency was surmounted by feeding with *Bacteroides fragilis* (*B. fragilis*), immunization with *B. fragilis* polysaccharides, or adoptive transfer of *B. fragilis*-specific T cells. Besides the effect of improving checkpoint therapies, *Bacteroides* groups are also correlated to resistance to the development of gastrointestinal and hepatic complications caused by immune checkpoint inhibitors. Dubin *et al.* showed that patients who developed new-onset, immune-mediated colitis caused by ipilimumab treatment had a lower level of *Bacteroidetes* than individuals without development of colitis after receiving anti-CTLA-4 [78]. Though *B. fragilis* groups exhibit promising potential for the development of specific immunomodulatory products for checkpoint immunotherapy, research is only limited to the *in vitro* experiment. Therefore, the immunomodulatory potential of *B. fragilis* must be demonstrated by *in vivo* animal models before advancing to clinical trials.

2.4. Probiotic

Probiotic, popularized by R. Fuller in 1989, was defined as "living micro-organisms that upon ingestion in certain numbers exert health benefits beyond inherent general nutrition" [79, 80]. Such a definition means that the probiotic organism can stay alive or temporarily colonize the intestine, which is a considerable mechanism to manipulate the intestinal microflora to elevate the populations of "beneficial bacteria". This probiotic-based manipulation of gut microflora inevitably influences the metabolism, immunologic function, digestion, and brain-gut interaction of the hosts [25, 81]. To date, diverse genera of microorganisms have been utilized as probiotics, such as *Lactic acid bacteria*, *Bifidobacteria*, *Enterococci*, and *Lactobacilli* [81], for the treatment of different diseases, including rotavirus infections, antibiotic-associated diarrhea, irritable bowel syndrome, inflammatory bowel

disease, atopy in at-risk infants, rheumatoid arthritis, and chronic sinusitis [82-85]. Recently, the development of culture-independent, high-throughput molecular techniques have provided unprecedented insight into the compositional diversity and functionality of intestinal microbiota, revealing the association of disorders with the disease-specific dysbiosis shifts in gut microbiota, such as colorectal cancer, type 2 diabetes and obesity [81]. Probiotic supplementation has been applied as a common approach to convey health benefits to modulate these disorders by modifying the gut microbiota [11]. For example, a preclinical outcome supported the "anti-obesity" effects of *Lactobacillus gasseri* (*L. gasseri*) BNR17 by suppressing the growth of adipocyte tissue, which is the primary source of leptin and adiponectin [86]. Kahouli *et al*. demonstrated the great potential of probiotics, *Lactobacillus fermentum* NCIMB-5221 and -8829, in inhibiting the growth of colorectal cancer cells and facilitating the reproduction of normal epithelial colon cell *via* the production of short-chain fatty acids [87]. Moreover, probiotics even promote the release of gastrointestinal hormone to modulate the behavior of central nervous system (CNS) through the bidirectional neuronal signaling pathway, which is a portion of microbiota-gut-brain axis [4]. The outcomes of clinical trials have confirmed the effect of probiotics on the CNS, suggesting that gut microbiota has a significant impact on human brain development function [88, 89]. For example, a daily dose of *Lactobacillus plantarum* (*L. plantarum*) WCFS1 to children with autism spectrum disorder could improve their academic performance and attitude towards food [90]. In addition, healthy volunteers treated with orally-administrated *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* (*B. longum*) R0175 in a randomized trialresulted in reduced psychological distress [91]. Dosages of a multispecies probiotic comprising *Lactobacillus brevis W, Bifidobacterium lactis* (*B. lactis*) *W, Lactobacillus acidophilus* (*L. acidophilus*) W37, *Bifidobacterium bifidum* (*B. bifidum*) W2, *Lactobacillus salivarius* W2, *Lactobacillus casei* (*L. casei*) W5, and *Lactococcus lactis* (*L. lactis*) (W19 and W58) to healthy people exhibited a remarkable decline in the cognitive response to sadness

[91]. However, probiotic-based tests involving patients encountered with anxiety and clinical depression are relatively rare, requiring more studies to verify this effect.

2.5. Magnetotactic Bacteria

Besides the bacteria mentioned above, magnetotactic bacteria, a type of gradient-inhabiting microorganisms, have also shown promising potential for disease treatment [92]. These magnetotactic bacteria possess an aerotaxis property, which enables them to compete at the oxic-anoxic interface. Such aerotaxis property is attributed to their biomineralized magnetosomes, a kind of magnetic nanoparticles wrapped in intracellular membrane containing permanent magnetic dipoles, which allows them to line up along magnetic field lines. This magnetically-forced alignment drives the bacteria to efficiently move toward a microaerobic niche with an optimal oxygen concentration, known as magneto-aerotaxis. Using these self-propelled bacteria, a number of therapeutic drugs can be transported to hard-to-treat oxygen-lacking regions within solid tumors, surpassing the diffusion limits of large drug molecules [93, 94]. In an interesting study, the *Magnetococcus marinus* strain MC-1 was able to deliver drug-entrapped nanoliposomes to hypoxic regions of the tumor *via* magneto-aerotactic migration [93]. Compared with the nanoliposome formulation, MC-1 cells carrying drug-entrapped nanoliposomes could penetrate hypoxic regions of the tumor more effectively under the magnetic guidance.

3. Genetic Engineering Advances Bacterial Properties

Although bacteria have unique capabilities for disease treatment, some key challenges must be addressed, such as biocompatibility, stability, and efficiency. To overcome these limitations, genetic engineering technology is employed to strengthen the traits of bacteria or confer other beneficial functions to bacteria since their genetics can be easily manipulated, permitting the design of “perfect” bacterial therapies [13]. For instance, *E. coli* has been used

for disease treatment due to its ability to preferentially survive in hypoxic tumors and initiate immune responses. Genetic engineering endows *E. coli* with additional abilities, including targeting disease sites and other related organs, accumulating and breeding in specific tissues, and secreting therapeutic proteins [17]. Using genetic engineering technology, *in vivo* behaviors of bacteria can be well-regulated to obtain more potent therapeutic effects.

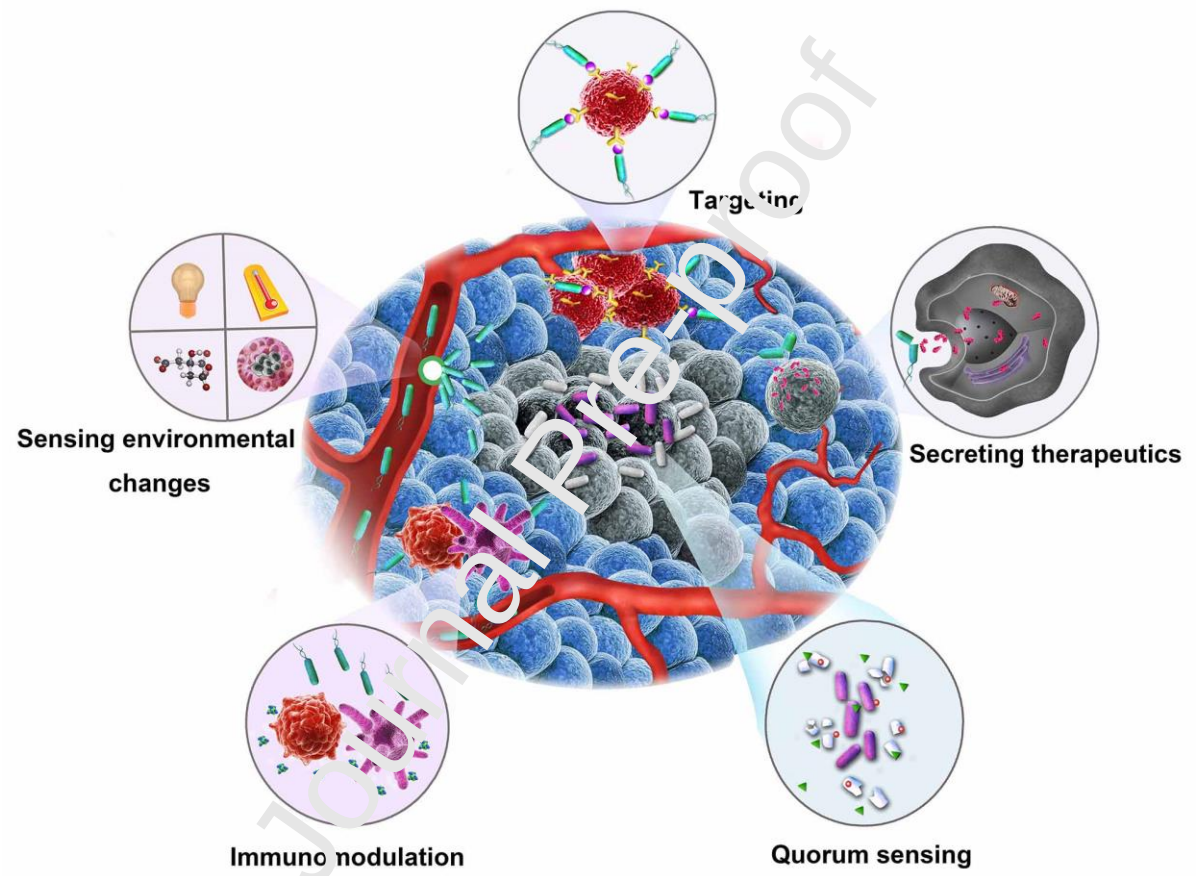

Fig. 3. An overview of engineering bacteria for therapeutic applications. A series of strategies, such as quorum sensing, targeting, expressing and releasing therapeutics, sensing environmental changes, and immunomodulation, have been genetically designed to regulate the bacterial behaviors for enhanced effect of disease treatment.

Conventional approaches of genetic engineering contribute limited modifications to inherent bacterial functions. Recently, synthetic biology, a rapidly developing discipline aiming at reasonably designing the behaviors of living microorganisms, has become an

important strategy for programming the behavior of bacteria with more complexation and precision [95]. Such synthetic biology technique makes use of bacteria as a modular platform for engineering, in which genes and promoters can be interconverted and incorporated to create nuanced and complex circuits. This kind of circuit could control the group behavior of the bacteria, coordinate their activities and initiate responses to the disease [18]. Meanwhile, these engineered bacteria could be eliminated from the host once the condition is alleviated [18]. In this part, the mechanisms of genetic circuits and genetic engineering for advancing bacterial properties (**Fig. 3**), including manipulation of quorum sensing, promotion of targeting capacity, sensing of environmental changes, production of therapeutics, and stimulation of the immune system, will be introduced.

3.1. Quorum Sensing

Bacterial quorum sensing refers to a behavior of transcribing specific genes when the extracellular chemical signals produced by bacteria themselves accumulate in the surrounding environment up to a threshold. This phenomenon was first identified in the 1980s, by the landmark discovery that the *luxI* gene produced *N*-3-oxohexanoyl-L-homoserine lactone (3OC6-HSL), which bound with the *luxR* transcriptional activator upon reaching a threshold concentration, leading to the transcription of *luxI* operon and bioluminescence [96-98]. In the later 1990s, the development of DNA sequencing and comparative sequence analysis facilitated a sharp increase in discoveries of other bacterial genes similar to *luxI*-*luxR* systems for conjugation, exoenzyme production and antibiotic synthesis [99]. Soon afterwards, the quorum-sensing signal molecule in *Salmonella pneumoniae* strains was found as a small peptide (often referred to as a pheromone) [100], while *Staphylococcus aureus* (*S. aureus*) utilized small cyclic peptide pheromones to stimulate the transcription of genes encoding extracellular toxins [101]. Based on these studies, genetic circuits consisting of an activator and a series of operons were generated in synthetic biology to manipulate the behavior of the

bacterial quorum sensing [31, 102, 103]. Jeff Hasty's group focused on the design of quorum-sensing circuits that controlled the bacterial gene expression responding to changes of population density [102, 104, 105]. In a recent study, they designed a quorum-sensing genetic circuit using *E. coli*, consisting of three constituents including *luxI*, *luxR* and acyl-homoserine lactone (AHL) [104]. In this cycle, AHL was produced by enzymatic reaction of LuxI and rapidly diffused out to the extracellular environment, maintaining a reasonable level when the population density was low. However, when the population density of bacteria raised to a certain threshold, the excess AHL would accumulate in the cell and combine with its receptor protein LuxR to activate a promoter P(*luxI*) that drove the transcription of target genes. As a result, this quorum-sensing genetic circuit could generate synchronized oscillations in a growing population of cells.

The attractive characteristics of quorum-sensing circuits have facilitated their integration as a versatile module for density-dependent bacterial therapies. For example, Swofford *et al.* incorporated nonpathogenic *Salmonella* with a density-dependent quorum-sensing switch, which initiated drug expression only in the close-packed colonies in tumors [106]. Such quorum-sensing switch was comprised of the *lux* quorum-sensing system and a GFP reporter. The *luxI* produced the communication molecule 3OC6HSL, which bound to LuxR protein to induce the p(*luxI*) promoter controlling the transcription of *luxR*, *GFP*, and *luxI*. When the bacterial density increased, the concentration of 3OC6HSL in the cell raised synchronously, forming a positive feedback loop which elevated the expression of *GFP*. Due to the aerotaxis of *Salmonella*, the quorum-sensing bacteria only drove the *GFP* expression in high-density colonies within tumors. Din *et al.* created another genetic circuit, which achieved the synchronized and periodic release of a bacterial toxin in attenuated *Salmonella enterica* subsp. *enterica* serovar Typhimurium strains (**Fig. 4**) [30, 107]. In this genetic circuit, p(*luxI*) promoted the transcription of genes encoding four constitutes including *luxI*, the drug, a fluorescent protein for reporting the dynamic of bacterial density, and a lysis protein called

φX174E from bacteriophage. As the bacterial population increased up to the concentration threshold, the transcriptional procedure driven by p(*luxI*) promoter was initiated in almost all cells, resulting in drug expression and release because of the lysis-triggered disruption of bacterial cells. A few population survivors repopulated, leading to cyclical bacterial lysis and drug delivery. After treating the tumor-transplanted mice with these programmed bacteria, a dynamic of synchronized periodic bacterial population was observed. Anderson *et al.* linked the capacity of invading mammalian cells with the bacterial population density by placing invasin as an output module under the control of a quorum-sensing genetic circuit [95]. By creating a quorum-sensing plasmid where *inv* gene encoding invasin was fused to the downstream of p(*luxI*) promoter, invasin was expressed by a constitutive promoter, enabling *Salmonella* to invade the tumor cells dependent on the cell density of bacteria.

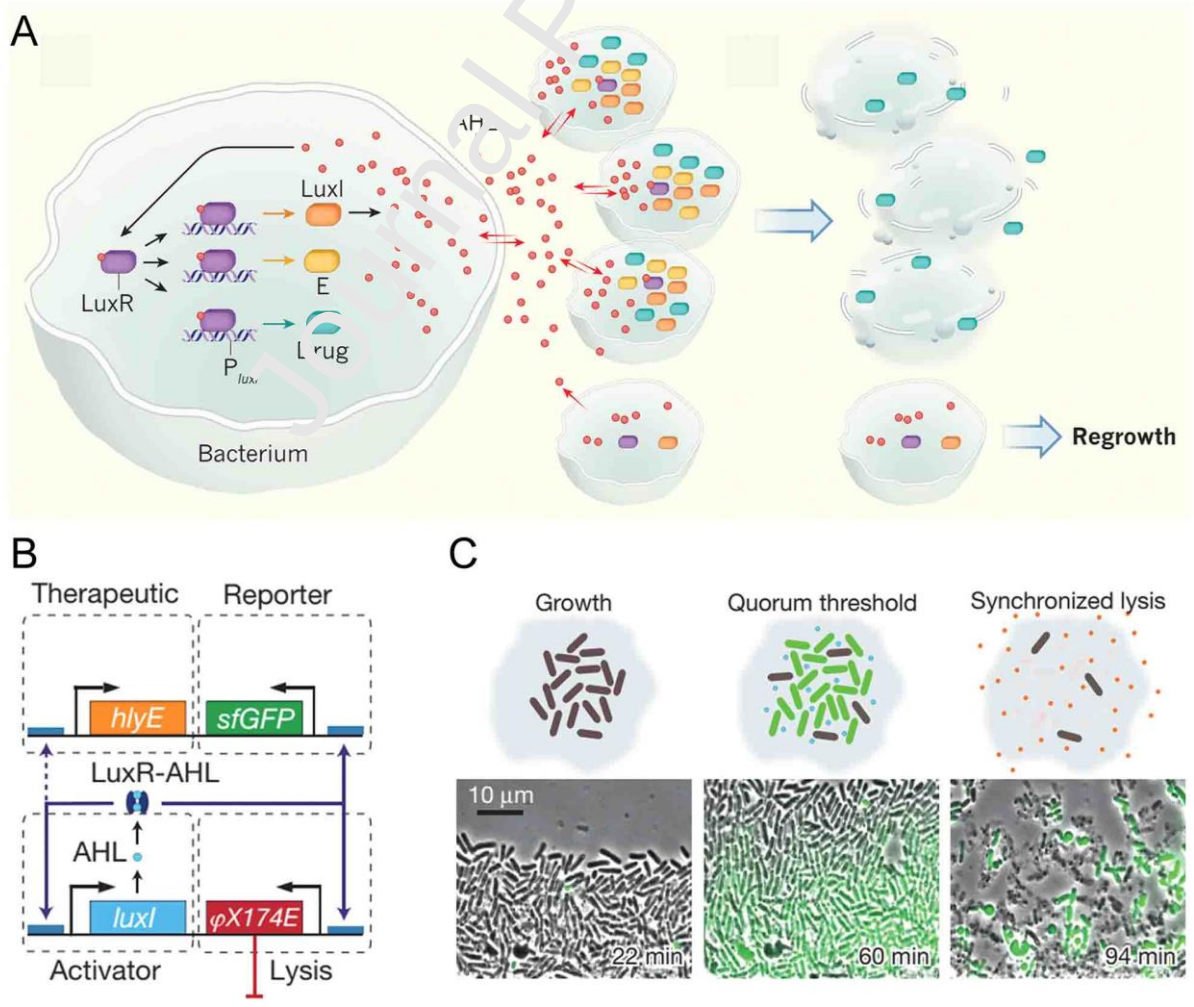

Fig. 4. The construction and mechanism of engineered bacteria with synchronized lysis for drug delivery. (A) The diagram of synchronized cyclical lysis and drug release of these engineered bacteria with this type of lysis circuit *in vivo*. (B) The construction of circuit containing an activator and lysis plasmid. Upon the AHL concentration reaching a threshold, the *luxI* promoter was activated to transform *luxI*, gene E for lysis, and *sfGFP* or *luxCDABE* as the reporter module. (C) Typical time-series schematic descriptions and images showing the three main growth stages of circuit-harbouring bacteria. Reproduced with permission [60, 107]. Copyright 2016, Springer Nature.

Moreover, quorum-sensing circuits are also edited to synchronize their gene expression upon sensing environmental inducers. Saeidi *et al.* presented a synthetic genetic system, which comprised of the devices of quorum sensing, killing, and lysing, enabling *E. coli* to detect and kill a pathogenic *Pseudomonas aeruginosa* strain by producing and releasing pyocin [108]. In this system, the *tetR* promoter, which was constitutively switching on, initiated the expression of a transcriptional factor, LasR, to bind with $3OC_{12}HSL$ produced by *Pseudomonas aeruginosa*. The formed LasR-$3OC_{12}HSL$ complexes would induce the *luxR* promoter to express toxic pyocin S5 and lysis E7 proteins within the *E. coli*. Once the concentration of lysis E7 protein rising to a threshold, the *E. coli* cell membrane was disrupted to liberate the accumulated pyocin S5, followed by diffusion to the targeting pathogens and damaging their cellular integrity to kill them. Wu *et al.* rewired the autoinducer-2 (AI-2) signaling pathway of *E. coli* to guide them towards a squamous cancer cell line of head and neck (SCCHN), in which the expression of a drug was initiated dependent on the density of epidermal growth factor receptor (EGFR) [109]. The SCCHN was targeted by “nanofactory”, a fusion protein containing an EGFR antibody and AI-2 synthase. After binding to EGFR overexpressed on the surface of SCCHN, synthesized Al-2 molecules were discharged from the cell surface and sensed by engineered *E. coli*, which migrated to the signal-generating site

and decided the initiation of gene expression depended on AI-2 level proportional to the EGFR surface density.

3.2. Surface Properties and Targeting Capacity

Bacteria can spontaneously enter tumors and colonize the hypoxic core, an immune-privileged environment that protects them from elimination by immune cells like macrophages or neutrophils [13, 58]. This natural targeting process can be potentially amplified by augmenting other targeting mechanisms. Genetical engineering of bacteria to display tumor-targeting motifs on their outer membrane is one of the targeting methods. These targeting motifs include antibodies binding to upregulated receptors on cancer cells, synthetic adhesive molecules mimicking immunoglobulin fragments or recognizing antigens, and tumor-localizing peptides like RGD [110-112]. Lambea *et al.* constructed an *E. coli* strain with synthetic adhesins (SAs) constitutively and stably expressed on the surface, directing a specific adhesion of bacteria to surfaces or cells expressing corresponding antigens [112]. Administration of bacteria expressing SA molecules to tumor-transplanted mice resulted in a more effective bacterial colonization in tumor tissues compared with wild-type bacteria. Such SA-anchoring strains provided a modular platform for targeting localization to a range of tumors by using different SAs. These tumor-targeting strategies have a remarkable influence on the successful clinical translation of bacterial therapies.

3.3. Sensing Environmental Changes

Bacteria can also be engineered to respond to various stimuli, such as specific molecules, light, hypoxia, temperature, and radiation, in the environment. When the surrounding conditions change slightly or moderately, the stimuli-responsive bacteria will exhibit dramatic

changes in behavior, thereby limiting their function within specific situations [113]. In this part, we will review some examples of bacterial therapies engineered with stimuli response.

Bacteria have been engineered to sense the glucose gradient, a tumor environment cue. As a typical example, a previously-reported synthetic hybrid receptor, comprising the periplasmic domain of the Trz1 chemotactic receptor [114], was incorporated into the tumor-targeting *E. coli* to trigger GFP expression dependent on the glucose concentrations [115]. This Trz1 receptor-equipped bacteria could report the concentration gradient of glucose over tumor cell masses in a microfluidic chamber by the expression of GFP. This method showed the potential to characterize glucose profiles and metabolic activity in different tumor types.

As mentioned previously, obligate and facultative anaerobic bacterial cells are naturally capable of sensing the oxygen concentration in surroundings and accumulating in the hypoxic region of solid tumors. Another hypoxia-sensing approach is utilizing the oxygen-sensitive promoters, including the synthetic FF20, the endogenous promoter *pepT* or formate dehydrogenase (*fdhF*), which were sequenced and identified by several groups [116, 117]. By placing essential genes under the control of these hypoxia-sensitive promoters, the bacteria were engineered to produce a therapeutic molecule or express an essential gene only in the hypoxic tumor area, achieving targeted delivery of therapeutic drugs [95, 118, 119]. Anderson *et al.* genetically engineered *E. coli* to express invasin proteins to achieve specific invasion of mammalian cells. The gene encoding invasin was deposited under the management of a hypoxia-sensitive promoter with the *lux* quorum-sensing circuit of *Vibrio fischeri*, ensuring the implementation of cellular invasion only within hypoxic tumor environments [95]. Similarly, Yu *et al.* developed the bioengineered *S. typhimurium* strain SL7207 with the *asd* gene encoding the expression of diaminopimelic acid (DAP), an essential component of the bacterial cell wall, under the control of a hypoxia-responsive promoter p(*pepT*). With the specific expression of DAP in the hypoxic conditions, these engineered *Salmonella* survived only in oxygen-lacking tumor environment without influencing their original functions [118].

Ion channel is a kind of pore-creating membrane protein that controls the flow of ions across the cell membrane and regulates the cell volume. Currently, photosensitive ion channels have been developed in bacterial systems using genetic engineering to achieve a novel transcriptional control of cell functions using light [120]. Motta-Mena *et al.* exploited a bacteria-based light-dependent or optogenetic drug delivery system using a bacterial light-oxygen-voltage protein EL222, which was combined with DNA upon stimulation with blue light [121]. Under the control of blue light, a broad dynamic range of protein expression as well as a kinetic of protein activation and deactivation were observed *in vitro*. This type of bioengineered bacteria was further developed to trigger gene transcription in diverse eukaryotic cells under the control of blue light illumination. Moreover, genetically-encoded optogenetic manipulation is also an important method for modulating cellular protein-protein interactions (PPIs) due to their noninvasiveness, high PPI activation rate, invertibility, and reduced adverse effects. Kaberniuk *et al.* reported a reversible light-inducible manipulation of the interaction between bacterial phytochrome BphP1 and its receptor PpsR2 in *Rhodopseudomonas palustris* strain [122]. The BphP1-PpsR2 binding was extensively characterized in mammalian cells, followed by employment to transfer targeting proteins to specific cellular compartments like the plasma membrane and the nucleus. The results indicated that light-induced regulation of cell morphology led to a substantial increase of the cell area.

In addition to stimuli such as chemical molecules, hypoxia and light mentioned above, radiation and temperature have also been examined to induce the gene expression in bacterial therapies. One method is using γ-irradiation to indirectly activate the inducible *recA* bacterial promoter [123-125]. By placing the targeting gene under the control of *recA* bacterial promoter, the DNA damage caused by γ-irradiation would subsequently facilitate the degradation of the *RecA* repressor, LexA, thereby activating promoter *recA* to allow the transcription of downstream genes. The usage of gamma irradiation could achieve deep

penetration in tumor tissues. However, the gamma irradiation might also cause damage to adjacent healthy cells and probably trigger undesired mutations to bacterial genes encoding therapeutic molecules. Temperature is also employed as another unique input signal in genetically-engineered bacterial therapies to sense and respond to internal environments or external stimuli like focused ultrasound. Piraner *et al.* presented two thermally-dependent transcriptional repressors to switch the bacterial gene expression within a threshold range of 32-46 °C [126]. These bio-switches were integrated with thermal gene circuits and applied in three *in vivo* treatment options consisting of spatially-precise activation by focused ultrasound, regulation of activity responding to host fever, and self-destroying after fecal excretion to avoid environmental evasion.

3.4. Secreting Therapeutics

Another advantage of bacterial therapeutics is focused on the transformation of genes encoding therapeutic molecules to increase their effectiveness. To date, several major categories of bacteria-engineering methods have been investigated, including expression of functional drugs or molecules, expression of enzymes to convert a prodrug, and delivery of therapeutic DNA or RNA to target cells [15]. The selection of bacterial genus or genetic circuits is dependent on the targeting process and the site of function. These strategies have been tried in clinical tests to treat a range of diseases, including cancer [71, 127-134], inflammatory disease [34, 36, 135-137], oral mucositis [138], dental caries [139], diabetes mellitus [37, 140-144], gastrointestinal infections [145-149], HIV infection [150-152], obesity [153], allergies [154], and hypertension [155]. For instance, several groups attempted to engineer the *E. coli* or *S. typhimurium* to express Cytolysin A (ClyA, also known as HlyE), a bacterial toxin that formed pores on mammalian cell membranes to induce cellular apoptosis, to treat tumors [156-158]. The mice showed reduced tumor growth after receiving ClyA-expressing bacteria. For type 1 diabetes, oral administration of *L. lactis* secreting either

proinsulin or GAD65, combined with cytokine IL-10, revealed prevention and reversion of β-cell damage in a mouse model with type 1 diabetes mellitus [140-142]. These bioengineered bacteria have undergone clinical-grade productions and trials, laying the foundation for future clinical tests. Moreover, *L. lactis* secreting IL-10 was orally delivered to reduce inflammation in mouse colitis models [34]. This engineered *L. lactis* achieved equivalent results with an estimated >10,000-fold lower IL-10 exposure compared to a systemically delivered recombinant protein, providing a promising platform for bacteria-based therapeutic protein delivery with higher efficiency and fewer side effects.

Although bacteria could express an extensive range of therapeutic agents, an advancing challenge is to effectively release the therapeutic molecules from bacteria into the microenvironment. To solve this problem, two methods have been employed, including secretory release and lytic release [17]. The secretory release is implemented using a leader signal sequence, which is a short peptide linked to the N-terminus of the target proteins [71, 73, 127]. This leader signal sequence is similar to zip code, which transports the translocated proteins to the bacterial periplasm and secretes them out from the cell [159]. However, the secretory release of delivered protein is always dependent on their secretory pathways, limiting their application within certain microorganisms (*e.g.*, *E. coli* doesn't spontaneously secrete proteins) [160]. Lytic release relies on the expression of specific phage lysis genes or the usage of additional antibiotics to damage the bacterial structure, realizing the release of secretory proteins from bacteria [123, 161]. Gahan and co-workers lysed bacteria using additional ampicillin to liberate plasmids for uptake by tumor cells [52]. In addition, better cell lysis was also achieved by placing an adaptive bacteriophage lambda lysis operon under the control of a tetracycline-induced promoter [162].

3.5. Immunomodulation

Immunomodulation therapy or immunotherapy harnesses the host's immune system for the management of diseases by inducing, enhancing, or suppressing the immune response. The bacteria-induced immune response was first investigated in the late 19th century by Coley, who observed tumor regression on some of his patients after infection with bacteria [163, 164]. Subsequently, he set out to create a bacterial mixture with more safety comprised of heat-inactivated *Streptococcal* microorganisms and *Serratia marcescens*, later known as Coley's toxin [165]. With the uncovering of the mechanisms of bacteria involved in the host's immune responses, the use of live bacteria offers more exciting possibilities in immunotherapies [166-170]. To date, bacterial immunotherapy has been utilized as an essential approach for the treatment of cancer and immune disorder-related diseases, such as food allergies, inflammatory bowel disease, and asthma [171, 172].

Despite the advancement of bacterial immunotherapies against various diseases, a few patients treated with bacteria suffered from systemic infections and eventually died [173]. To overcome these barriers, gene engineering technology is employed to construct attenuated bacteria with low infection capabilities. For example, *Salmonella* has been manipulated with some genetic alterations, aiming at producing an attenuated strain that can be safely applied to human bodies while maintaining their natural capacity of tumor targeting. *S. typhimurium* VNP2009 is a commonly-used attenuated strain deposing two genes, called *msbB* and *purI* [44, 65]. The knock-out of these genes prevents their duplication in normal organs, like liver and spleen, while introducing the requirement of external sources of purines provided by the external media of tumors [174].

Moreover, bioengineering of bacteria with foreign genes to express antigens or antibodies can also strengthen the abilities to induce adaptive cellular immune responses [175-177]. For example, attenuated *Listeria monocytogenes* (*L. monocytogenes*) carrying a gene for antigen expression has been an attractive strategy for vaccine development owing to its intrinsic features, including infection of antigen-presenting cells and the mucosal route of infection.

Gunn *et al.* constructed a recombinant *L. monocytogenes* strain (Lm-LLO-E7) secreting a fusion protein integrating the human papilloma virus-16 (HPV-16) E7 protein with a nonhemolytic listeriolysin O (LLO) [129]. The Lm-LLO-E7 effectively induced the suppression of the E7-expressing TC-1 tumor, bearing on syngeneic C57BL/6 mice. These *L. monocytogenes* were also engineered to envelope HIV-related antigens to elicit sustained high levels of HIV-specific cytotoxic T lymphocytes in mice, serving as an HIV vaccine [53, 178].

In addition, live bacteria have also been engineered to deliver immunomodulatory proteins to enhance the immune response [71, 73, 74, 179]. Trefoil factors (TFFs)-secreting *L. lactis* were found with higher efficiency in alleviating intestinal inflammation compared with oral or rectal administration of purified recombinant TFFs [137]. A similar study showed that *L. lactis* secreting anti-tumor necrosis factor (TNF) nanobodies or IL-27 had a greater inhibitory effect on intestinal inflammation in mice than systemically-administered proteins [35, 36]. In a recent study, Ohkouchi *et al.* engineered recombinant *L. plantarum* NCL21 to express Cry j 1, a typical Japanese cedar pollen allergen, followed by orally delivering Cry j 1-*L. plantarum* to a murine model of cedar pollinosis [180]. The results showed that this vaccine could effectively ameliorate cedar pollinosis-like clinical symptoms and allergen-specific IgE responses.

4. Biomaterials for Oral delivery of Bacteria

Up to now, oral intake of bacteria has demonstrated excellent clinical applications, such as probiotics, for the treatment of lactose intolerance and some allergic reactions [25, 181]. However, oral administration of bacteria always suffers from low bioavailability, which is caused by several obstacles, including the acidic environment in the stomach, pre-systemic degradation by enzymes, and poor permeability in the intestinal mucosa. The strong acidity in the stomach results in the inactivation of bacteria. The existence of massive enzymes, including the gastrointestinal cavity, intestinal wall enzymes, bacterial enzymes, and hepatic

enzymes, should be responsible for the degradation of bacteria [182]. Additionally, the poor penetration of the intestinal mucosa also shorts the retention time in the gastrointestinal tract, reducing the efficiency of the therapeutic effect [182]. To help the bacteria overcome the peracid environment in the stomach, maintain their integrity in the gastric fluid, protect them from enzymatic degradation, and finally reach the intestinal tract, encapsulation technology has been used to embed the bacteria in specific protective biomaterials. It is essential to select appropriate biomaterials as the encapsulating matrix to make a significant difference in permeability, mechanical stability, pH sensitivity, and bacterial release rate to improve the viability of bacteria in the gastrointestinal environment [5, 23].

The choice of encapsulation material is a critical factor in protecting bacterial viability. Firstly, the materials should possess excellent biocompatibility and biodegradability to ensure the safety of the host and bacteria [21]. Secondly, the encapsulation materials should be able to tolerate an acidic environment to guarantee the integrity of the capsule in the gastric fluid. Additionally, it is also important that the method of encapsulation should be mild enough to minimize damage to the entrapped cells. To date, various polymeric materials including alginate, k-carrageenan, xanthan gum, gellan gum, Eudragit, starch derivatives, cellulose, casein, poly(L-lysine) (PLL), pea protein, whey proteins, and pectin have been used to encapsulate bacteria for oral administration. In this part, which focuses on the encapsulation of therapeutic bacteria for disease prevention, diagnosis, and treatment, we summarize the substrates used alone or in combination for encapsulating bacteria. The frequently used biomaterials and encapsulation technologies for oral delivery of bacteria are listed in **Table 2**. The structures of commonly used encapsulation materials are listed in **Fig. 5**.

Table 2. Overview of the biomaterials and techniques used for bacterial encapsulation for oral delivery.

Materials	Bacteria	Encapsulating	Carrier	Reference

		technology	formulation	
Pectin-starch	*L. plantarum*	Extrusion	Core-shell	[183]
Cellulose-alginate	*L. plantarum*	Extrusion	Core-shell	[184]
Chitosan-alginate	*Bacillus coagulans*	Layer-by-layer	Core-shell	[185]
Ca-alginate/protamine (CAP)	*L. casei* CICC 23185	Extrusion	Core-shell	[186]
Alginate-chitosan-alginate (ACA)	*Escherichia coli* DH5	Layer-by-layer	Particle	[187]
Alginate-poly-L-lysine-alginate (APA)	*L. plantarum 80*	Layer-by-layer	Particle	[32]
Cellulose-Ca-alginate	*L. plantarum*	Extrusion	Core-shell	[188]
Amorphous silica-alginate	*L. rhamnosus* GG	Biomimetic mineralization	Core-shell	[189, 190]
Ethylenediaminetetraacetic-calcium-alginate (EDTA-Ca-Alg)	*L. rhamnosus* ATCC 53103	Emulsification	Core-shell	[191]
Alginate-fenugreek gum-locust bean gum	*Lactic acid bacteria*	Extrusion	Core-shell	[192]
Fat sodium caseinate (FSC) Sodium caseinate (SC)	*L. paracasei* F19, *L. casei* BFLM218	Emulsification	Particle	[193]
Alginate-milk	*L. bulgaricus*	Emulsification	Particle	[194]
β-glucan	*L. bulgaricus*	Extrusion	Core-shell	[195]
Alginate-cellulose nanocrystals (CNC)-lecithin	*L. rhamnosus* ATCC 8395	Freeze drying	Particle	[196]
Liposome	*E. coil*	Inverse emulsion	Core-shell	[197]
Alginate-chitosan	*B. longum*	Surface coating	Multi-layer coating	[198]
Eudragit, microcrystalline cellulose (MCC), calcium-crosslinked alginate, and lactose	*L. casei*	Enteric coating	Core-shell	[199]
Poly (D,L-lactic-*co*-glycolicacid) (PLGA) alginate multiparticulate gels	*BiMuno's* GOS	Emulsion	Particle	[200]
Pea protein-polysaccharide (sodium alginate, iota-carrageenan and gellan gum)	*B. adolescentis*	Extrusion	Core-shell	[201]
Alginate-chitosan	*B. breve*	Layer-by-layer	Multi-layer coating	[202]
Xanthane gum-chitosan hydrogels	*P. acidilactici*	Extrusion	Core-shell	[203]
Maize starch	*L. plantarum* 299v	Freeze drying	Particle	[204]
D-glucose-alginate	*L. plantarum* *L. rhamnosus* *B. animalis subsp*	Emulsification Surface coating	Core-shell	[205]
Eudragit L100-55	Live bacterial cells	Lamination method	Polymer film laminate (PFL)	[206]

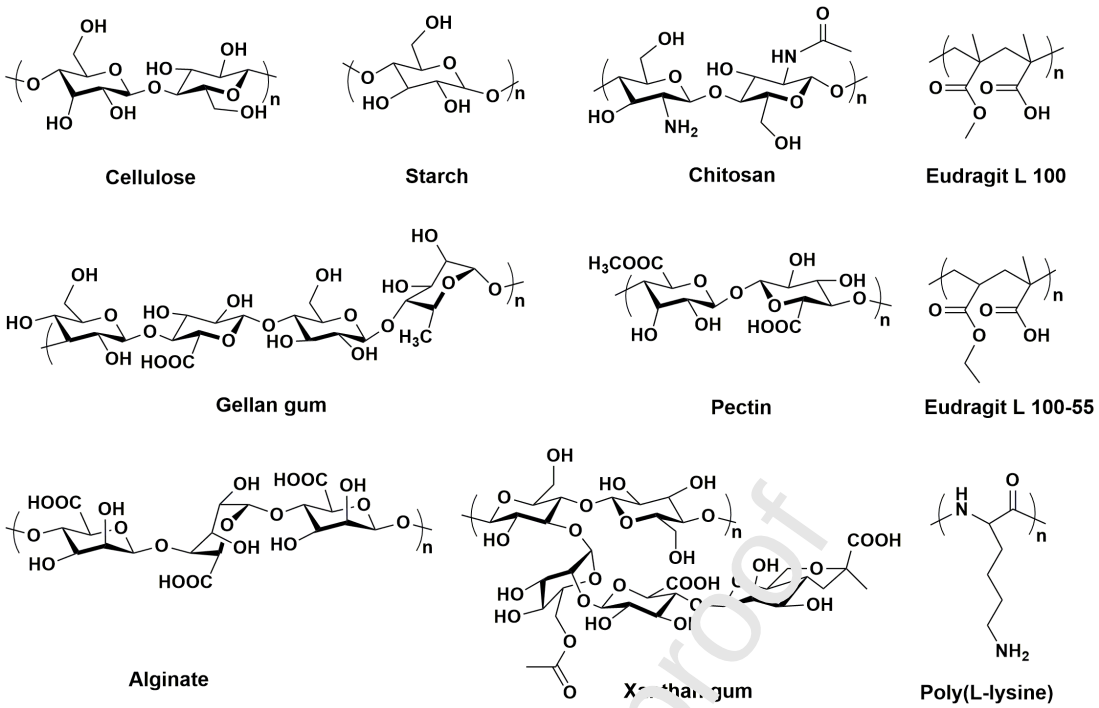

Fig. 5. Chemical structures of commonly used biomaterials for bacterial encapsulation.

4.1. Eudragit

Eudragit polymer generally refers to a series of polymethacrylates that are produced by dimethylaminoethyl methacrylates, methacrylic acids, and methacrylic acid esters in various ratios [207]. The coating of Eudragit polymer can exclude stomach acid but dissolve as pH rises in the intestine, protecting encapsulated drugs from degradation by gastric fluid and releasing them rapidly from microcapsules in the intestinal fluid [199]. Therefore, the Eudragit polymer is always used as an enteric polymer for the oral delivery of bacteria, endowing bacteria with several capacities, such as pH-dependent release, dissolution and bioavailability enhancement, sustained release, and colonic targeting [207]. For example, de Barros *et al.* used Eudragit coating to prepare a bacteria-encapsulated intestine-targeting carrier with a core-shell structure [199]. In this study, live *L. casei* was mixed with microcrystalline cellulose, calcium-crosslinked alginate and lactose into a granulation liquid, followed by coating with Eudragit through extrusion technology. The increased pH in the intestine induced the hydrophobic Eudragit to be hydrophilic, subsequently releasing the bacteria to targeted sites. As a result, Eudragit coating could protect dried live cells from the

acidic stomachic environment and guarantee the rapid release of cells in the intestine. Similarly, they used Eudragit L 100-55 to design a unique formulation called polymer film laminate (PFL) for oral delivery of live bacterial cells [206]. Eudragit L 100-55, an anionic copolymer of methacrylic acid and ethyl acrylate, could dissolve at pH 5.5 in the duodenum [207]. Probiotics were dried straightforward on a cast film of Eudragit L 100-55, followed by lamination to generate an oral dosage form. The experimental results showed PFL prepared with Eudragit alone favorably protected dried probiotic or live bacterial cells against simulated gastric fluid for 2 hours, and then released all living cells within 60 min of transfer to the simulated intestinal juice.

4.2. Polysaccharides

Polysaccharide and polysaccharide derivatives such as alginate, chitosan, cellulose, and starch, are accessible materials for encapsulating bacteria because of their excellent biocompatibility, biodegradability, and low cost.

Alginate, a family of linear unbranched saccharides containing different amounts of 1,4'-linked β-D-mannuronic acid and α-L-guluronic acid residues, is the most widely used natural polysaccharide for oral delivery of bacteria [208]. It is worth noting that, owing to the existence of carboxylic acid groups on monomer molecules, alginate is negatively charged when pH is higher than its pK_a (3.3-3.5). Moreover, alginate may gel upon contact with divalent metal ions (such as Ca^{2+}, Cd^{2+} or Zn^{2+}), formulating a so-called "egg-box structure" between four G residues [21]. Considering its acid-gel character, mild gelling condition, GRAS (generally recognized as safe) status, and non-toxicity, this polysaccharide polymer is extremely well suited for bacterial encapsulation [209]. Studies thus far have demonstrated the beneficial properties of alginate as a vehicle for the enteric delivery of bacteria. Various studies have been reported to encapsulate bacteria with alginate using extrusion and the emulsion methods, demonstrating the enhanced viability of a broad range of host cells,

including *Lactobacilli*, *Bifidobacteria*, and a probiotic yeast (*Saccharomyces boulardii*) in an acidic environment [210-216]. However, encapsulation of bacteria using alginate alone has limited loading capacity due to its weak supporting property, and microcapsules of alginate formation exhibit uncontrollable swelling behaviors. To achieve a more desirable effect, alginate is usually combined with other materials to encapsulate bacteria. For example, cellulose microgels with excellent porous structure had a higher loading capacity for embedding bacteria. Therefore, the integration of cellulose microgel core with alginate shell resulted in better acid resistance and probiotic viability. This core-shell gel could achieve sustainable release of *L. plantarum* cells for at least 360 min in simulated intestinal fluid without loss of viability, significantly longer than Ca-alginate gels [184]. Besides, porous silica can also combine with alginate to form core-shell encapsulation systems, which allow bacteria to survive in the stomach environment [189].

Chitosan, a natural linear cationic polysaccharide comprising both glucosamine and N-acetyl glucosamine residues, presents a range of benefits for drug delivery such as biodegradability, low toxicity and biocompatibility [208, 217]. As the amine residues on chitosan present a pK_a around 6.5, chitosan acts as a cationic polyelectrolyte due to the protonation of amino residues in the solution at pH below its pK_a [218]. Owing to this cationic property and the ability to tolerate acidic environments, chitosan has been considered as one of the most frequently applied materials for bacterial coating to protect them against the harsh gastrointestinal environment. Cook *et al.* accessed the protective effect of the chitosan coatings on probiotic-loaded alginate microcapsules while exposure to acidic conditions [219]. They found the coating of chitosan on *Bifidobacterium breve* (*B. breve*) NCIMB 8807 alginate microcapsules improved the survival of bacteria more effectively than the counterparts without chitosan coating. It was inferred that chitosan could behave as a buffer to reduce the permeability of microcapsules to acidic medium while maintaining its integrity, thereby enhance the tolerance of bacteria to the acid conditions. Similar results were

obtained by other studies, in which chitosan-coated alginate microcapsules improved the viability of encapsulated bacteria in gastric and intestinal media [220-226].

Other polysaccharides, such as pectin, xanthan gum, fenugreek gum, and locust bean gum, have also been used as encapsulation materials for oral delivery of bacteria [227, 228]. Pectin, a water-soluble biodegradable anionic polysaccharide, contains a linear α-(1-4)-D-galacturonic acid chain that is partly esterified by methoxy groups [228]. Dafe *et al.* loaded *L. plantarum* in a pectin-starch hydrogel using the extrusion method [183]. In this study, pectin was combined with starch to prevent the degradation of starch by pancreatic enzymes. These cells entrapped in pectin-starch hydrogel could remain stable in acidic or other adverse conditions. Xanthan gum, a heteropolysaccharide composed of poly-pentasaccharide groups formed from 2 glucose, 2 mannose and 1 glucuronic unit, possesses a similar property with alginate for bacteria encapsulation [227]. Ding *et al.* investigated the effect of encapsulating bacteria using xanthan gum, showing a higher protective level against bile and acid (at pH 2) conditions compared with that using alginate [229]. Xanthan gum was also combined with gellan to form acid-stable microcapsules [230]. The survival of *Bifidobacterium infantis* (*B. infantis*) and *B. lactis* encapsulated in xanthan gum-gellan microcapsules was vastly improved in the simulated gastric medium. Additionally, lactic acid bacteria encapsulated with alginate-fenugreek gum-locust bean gum matrix were also developed [192]. Compared to non-encapsulated bacterial cells, the encapsulated bacterial cells could overcome gastrointestinal transit and retain higher viability during freeze drying and storage conditions.

4.3. Poly(amino acids)

Poly(amino acid) like PLL and poly(L-ornithine) (PLO), as a type of natural cationic polymers, could complex with alginate *via* electrostatic interaction to form a microcapsule with a semipermeable membrane serving as a diffusion barrier [32, 231, 232]. The cationic PLL coating on the alginate gel can regulate the charge density and the pore structure on the

surface of alginate bead, modulating the eventual permeability of membrane structure [233]. Therefore, the alginate/PLL-based semipermeable membrane structure allows the transit of nutrients, secreted proteins and excretory products, meanwhile preventing the entry of adverse molecules or cells from the host, which could destroy the encapsulated bacterial cells [234]. Due to the very gentle, simple, and rapid immobilization process, alginate/PLL coating is a promising approach for bacteria encapsulation, which provides sufficient nutrient supplement and durable protection, ensuring the survival of the live cells during their passage. For instance, Chen *et al.* prepared alginate-PLL-alginate (APA) microcapsules, biometrics of alginate and PLL, for oral delivery of bacteria [32]. Results indicated that the APA microcapsules maintained the morphological stability under the simulated stomach conditions. However, APA microcapsules only protected bacteria in simulated gastric fluid for a very short time. The survival rate of encapsulated *L. plantarum* 80 decreased sharply after staying more than 5 minutes in a low pH solution. To prevent APA microcapsules from acid-induced inactivation and hydrolyzation by enzymes in the gastrointestinal tract, Ouyang *et al.* modified APA membranes and designed a novel multilayer alginate-PLL-pectin-PLL-alginate (APPPA) microcapsule to encapsulate live bacterial cells [235]. Compared to APA microcapsules, APPPA microcapsules could maintain excellent mechanical stability in simulated gastrointestinal fluid.

4.4. Proteins

Proteins, such as whey protein, pea protein, casein, and milk fat globule membrane proteins, have become a popular choice for encapsulation of probiotics or other live-cell substances, especially in the last few years. These biomolecules exhibit several unique structural and physicochemical properties for bacterial encapsulation, including the capability to bind with ions and molecules, gelation, pH-responsibility, interactions with other polymers, good biocompatibility and biodegradability, and controllable bioavailability of the bioactive

substance [21, 194]. Shi *et al.* reported a new alginate-milk microsphere to maintain the viability of *Lactobacillus bulgaricus* (*L. bulgaricus*) prepared by the extrusion method with a 100% encapsulation yield [194]. The results showed that the survival rate of *L. bulgaricus* entrapped in alginate-milk exhibited unnoticeable change when immersing in a simulated gastric juice at pH 2.5 for 120 min, suggesting the improved resistance to adverse conditions. Fat sodium caseinate and sodium caseinate microcapsules were found to enhance the acid tolerance of live cells, although these microcapsules were eventually digested in the murine stomach [193]. Moreover, both pea protein and whey protein were used to form a mixed matrix with alginate for oral delivery of bacteria [236, 237]. The coated probiotics not only showed a better survival ability in adverse environments than free cells, but also had a longer storage time [236]. Overall, these results indicate that proteins are playing an increasing role in transferring bacteria for oral administration.

4.5. Lipids

Phospholipids are the main components of cell membranes. Liposomes formed by phospholipid bilayers are often used as carriers for drug delivery because of their hypotoxicity, biocompatibility and easy modification with targeting ligands [197]. However, there is a lack of straightforward methods to sheathe the living microorganisms using phospholipid bilayers. Chowdhuri *et al.* used an inverse-emulsion method to produce phospholipid-based giant unilamellar vesicles (GUVs), followed by evaluating the protection of encapsulated *E. coli* and yeast, respectively, in the gastrointestinal environment [197]. Such an inverse-emulsion method could generate large-sized GUVs for living cells, protecting bacteria from external protease degradation and harsh biological environments. In another case, Cao *et al.* used a set of lipid membranes to encapsulate gut microbes like *E. coli* through biointerfacial supramolecular self-assembly, which presented enhanced treatment efficacies in two murine models of colitis (**Fig. 6**) [238]. Meantime, both *S. aureus* and *Enterococcus faecalis* could be

coated with lipid membranes. Due to this coated lipid membrane, orally-administrated *E. coli* could significantly endure various extreme conditions in the stomach and showed almost three-fold higher viability than uncoated bacteria. Meanwhile, this lipid membrane could be dismantled upon targeting disease sites, resulting in less damage to bacterial mucosal adhesion, colonization, and proliferation.

In addition to these synthetic phospholipid bilayer-based microcapsules, natural cell membrane, such as the erythrocyte membrane, was recently reported to coat bacteria by the extrusion method [239]. With the coating of the natural cell membrane, bacteria showed a low inflammatory response; a reduced removal by macrophages, low retention in normal organs and almost unvaried viabilities, providing a unique tool for their biomedical applications.

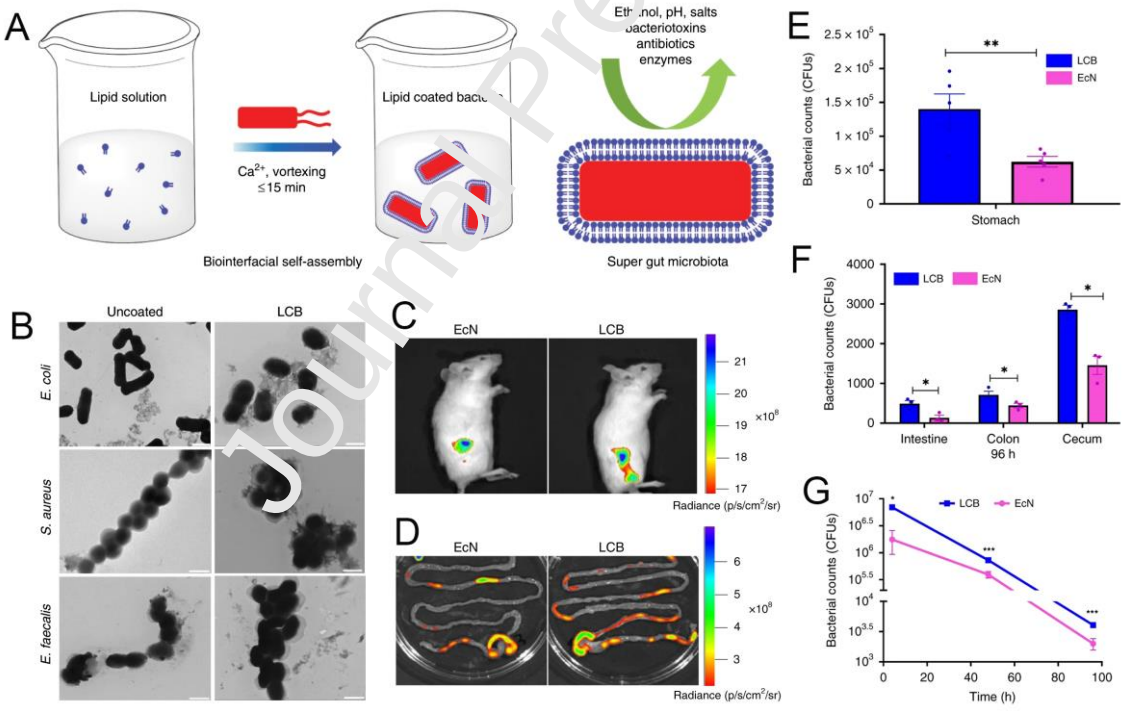

Fig. 6. Lipid membrane-coated bacteria (LCB) produced by biointerfacial self-assembly for improved efficiency of orally-delivered treatment. (A) The preparation of LCB by bio-interfacial supramolecular self-assembly. The lipid-based coating membranes protect bacteria from various harsh environments. (B) TEM images of unencapsulated bacteria (*E. coli*, *S. aureus* and *E. faecalis*) and LCB. Scale bar: 1 μm. (C, D) IVIS images of mice (C) and their

intestinal tracts (D) after oral administration of uncoated bacteria and LCB. (E, F) Bacterial survival in the stomach (E) and bacterial distribution in the intestine, colon, and cecum (F) after 96 h post-administration. (G) The comparison of total amount of uncoated bacteria and LCB retained in the intestinal tract. Reproduced with permission [238]. Copyright 2019, Nature Communications.

5. Emerging Technologies for Bacterial Encapsulation

Entrapping live bacterial cells into particles or beads for oral delivery results in an increase of bacterial viability by protecting them from adverse conditions, such as low pH, cold shock, bile salts, and heat shock. Although the intrinsic properties of various encapsulation materials offer protection for orally-delivered bacteria, the manners, in which these materials envelope bacteria, also influence their storage survival and behaviors in gastrointestinal environments. Conventional encapsulation technologies for producing bacteria-containing microcapsules include spray drying, spray cooling, spray coating, freeze drying, emulsification, coacervation, and extrusion. For oral delivery of bacteria, appropriate approaches can provide the required protection and increase the viability of bacterial cells. We summarize emerging bacteria encapsulation technologies in this section, ranging from traditional methods like extrusion and emulsification to recent attractive methods, including surface coating and microfluidics. **Table 3** shows a comparison of these encapsulation methods.

Table 3. A comparison of current popular encapsulation technologies.

Methods	Capsule sizes	Advantages	Disadvantages	References
Spry drying	5 μm -150 μm	1) Low cost 2) High production	1) High temperature may kill the cells	[24, 240]
Freeze drying	Not applicable	1) Suitable for heat-sensitive materials	1) High cost 2) Cell damage with eventual crystal formation	[240, 241]
Extrusion	2 mm - 5	1) Low costs 2) Minimal cell loss	1) Difficulty to scale up	[242]

	mm	3) High cell viability 4) Mild and simple preparation conditions		
Emulsion	25 μm - 2 mm	1) Easy to scale up 2) A high survival rate of probiotics	1) High material waste rates 2) Uneven size of capsules 3) Low productivity of prepared microcapsules	[242]
Layer-by-layer	Not applicable	1) Easy to manipulate 2) High reproducibility 3) High encapsulation efficiency 4) Control of the thickness of layer 5) Mild condition	1) The process is instantaneous 2) High time costing	[243]
Coacervation	Not applicable	1) High payload (99%) 2) The control of the release of core material 3) Suitable for heat sensitive probiotic bacteria	1) Difficulty in obtaining capsules with small sizes 2) Complexity of technique 3) High cost of the particle isolation procedure	[242, 244]
Biomimetic mineralization	Not applicable	1) High thermal and mechanical matrix stabilities 2) Capacity of resistance to harsh conditions	1) Small scale of application 2) Influence on the bacterial biological biosafety 3) Bulk encapsulation needs to be developed	[245, 246]
Interfacial polymerization on bacterial cell surface	Not applicable	1) The direct introduction of functional polymers for cell surface modification 2) High density of attached polymer	1) Rigorous preparation conditions 2) The complicated preparation process	[247, 248]
Microfluidic technology	Not applicable	1) Synthesis of monodisperse microspheres with precisely controlled size 2) High reproducibility 3) High encapsulation efficiency and low consumption of reagents	1) The application for bacterial encapsulation is under exploration	[249]

5.1. Conventional Methods

The conventional methods for bacterial encapsulation consist of spray drying, freeze drying, extrusion, and emulsification. Herein we will introduce these methods in the following parts.

5.1.1. Spray Drying

Spray drying is a conventional method for long-term storage in the food industry by transforming liquid solutions to dry powders [24]. Recently, this technique has been applied for the encapsulation of probiotic cells by spaying the mixture solutions containing a range of polymers, such as gelatin, whey protein isolate, gum Arabic, modified starch, maltodextrin/gum Arabic mixture, and β-cyclodextrin/gum Arabic mixture [237, 250-254]. Using this technology, small capsules with an average diameter of less than 100 μm can be generated with a comparably low cost and can be widely applicable in the food industry [24]. However, such technology requires bacteria with a high tolerance for heat, as high

temperature and rapid dehydration during spray can damage cell structure to induce high cell mortality, thus limiting its further application [255]. The air temperature of the chosen outlet is usually a compromise between the demanded residual water content and the survival rate of probiotics.

5.1.2. Freeze Drying

To date, freeze drying is the best way to dry bacteria from the aqueous dispersions to obtain them in a dried form while maintaining their viability [241]. In this technology, the aqueous solution of material containing the bacteria is first frozen, followed by sublimation of frozen solution under the chamber pressure [240]. However, the removal of frozen water during the freeze drying process will distort some polymer-based beads, leading to poor mechanical properties which may affect the survival of the encapsulated bacteria [196]. To overcome this limitation, some excipient polymers, such as cellulose nanocrystal (CNC) and lecithin, are added into the matrix [256-258]. Huq *et al.* reported that CNC exhibited an outstanding reinforcing effect to improve the poor mechanical properties of alginate matrix during the stabilization process of freeze drying [257]. Moreover, they also developed alginate-CNC-lecithin microcapsules to encapsulate and protect the probiotic bacteria during storage. Meanwhile, this alginate-CNC-lecithin microcapsule also provided a controlled release of bacteria into the intestine [196]. However, the energy costs of freeze drying are often enormous, hindering its use in large-scale processes.

5.1.3. Extrusion

Extrusion is the oldest method for preparing capsules with hydrocolloids due to its easiness, simplicity, low cost, and mild preparation conditions [24]. It only involves the processes of ejecting the bacteria-hydrocolloid mixture suspension through a syringe needle to form droplets and dripping into a hardening solution or setting bath [242]. Mei *et al.* applied a

coextrusion minifluidic method to develop an intestine-targeting carrier to deliver bacteria *via* the oral route (**Fig. 7**) [186]. In this method, the mixture of Ca-alginate and condensed probiotics was ejected through a syringe, followed by dripping into a hardening solution to obtain Ca-alginate beads (CA beads), subsequently absorbing protamine on their surface to form Ca-alginate-protamine beads (CAP beads) for oral administration. When CAP beads enter the stomach, the morphology of microcapsules remains stable. As microcapsules enter the intestine, the bacteria are released from the microcapsules. There are a variety of biomaterials, including cellulose-alginate, pea protein, tea protein, fenugreek gum, and locust bean gum, that have been used to encapsulate bacteria by extrusion [184, 192, 201, 236]. The size (2-5 mm) of capsules formed by the extrusion technique is dependent on the needle's diameter as well as the free fall height from the syringe needle to the fluid level of the alginate solution [259]. However, the main disadvantage of the extrusion method for bacterial encapsulation is the inability to create capsules smaller than 500 µm and larger than 3 mm. Meanwhile, the scale-up is difficult due to the slow generation of beads [260-262]. To overcome these challenges, some alterations have been developed, including the usage of nozzles to replace syringe and needle, or the utilization of emulsions to produce microcapsules.

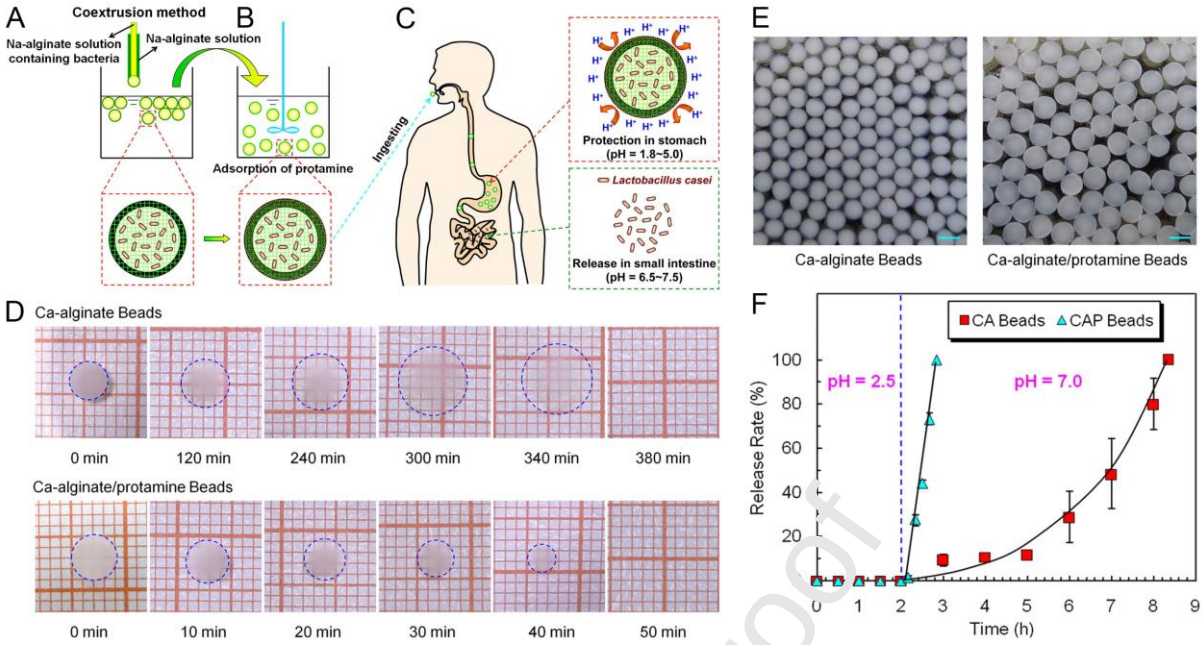

Fig. 7. Novel intestine-targeting Ca-alginate-based microparticles for protection and release of lactic acid bacteria. (A, B) Encapsulation of bacteria with alginate by (A) coextrusion to form CA beads and (B) surface adsorption of protamine to form CAP beads. (C) After oral administration, these capsules could maintain stability in the stomach while releasing bacteria in the intestine. (D) Photographs of the disassembling processes in intestinal fluids of pH 7.0. (E) Optical photographs of CA beads (up, 3.8 mm) and CAP beads (down, 4.3 mm) in the water at 25 °C. Scale bar: 4.0 mm. (F) The comparison of release rate in pH 2.5 and pH 7.0 between CA beads and CAP beads. Reproduced with permission [186]. Copyright 2014, American Chemical Society.

5.1.4. Emulsion

For bacterial encapsulation, the emulsion technique is the most suitable method involving flexibly controlling and adjusting the size of the consequent capsules [24, 242]. In this method, the aqueous hydrocolloid-cell mixture (uncontinuous phase) is emulsified into vegetable oil (continuous phase) including soybean oil, sunflower oil, canola oil or corn oil, followed by homogenizing to form water-in-oil emulsions. Upon the formulation of water-in-oil emulsions, the dispersed hydrocolloid-cell mixture will be insolubilized (*e.g.*, cross-linked) to obtain

small gel capsules in the oil phase, followed by harvest *via* filtration [259]. The choice of the insolubilization method is dependent on the category of supporting material used. Various materials, such as D-glucose, alginate, milk, lipid, β-glucan, sodium caseinate, and whey protein, can be used to form small droplets encapsulating live bacteria through emulsion technology [193, 195, 197, 205]. The main advantage of emulsification is the capacity to produce smaller capsules below 100 μm, compared to the extrusion technique [263, 264]. The eventual capsule size is controlled by the major parameters that influence the formation of the internal phase particle during routine emulsifying processes, such as the energy input during emulsification, the addition of emulsifiers, and the viscosity ratio between the dispersed and the continuous phase. Although emulsion methods have been widely used in bacteria encapsulation for decades, disadvantages, such as low yield of prepared microcapsules, high material waste rate, and uncontrollable size distribution, limit its robust application in large-scale production [242].

The four encapsulation methods presented above have their unique and specific features for bacteria encapsulation. The extrusion technique exhibits a vast diversity of machines and industrial components that are suitable for producing capsules using different mixtures of polymers and crosslinkers. In addition, this industrial equipment is also capable of creating capsules with a size that cannot be achieved by conventional protocols at a laboratory scale. Similarly, spray drying and freezing dry also present great flexibility in industrial production, although the process temperature is still a significant defect for spray drying and the energy costs are still a challenge for the use of freeze drying in large-scale processes. The emulsion method is the most-commonly applied bacterial encapsulating technology at the laboratory scale, which can produce capsules below 100 μm. However, more efforts are required for the application of the emulsion method on a large scale.

5.2. Surface Coating

Although current bacteria microcapsules produced by traditional encapsulation methods (*e.g.*, spray drying, freezing drying, extrusion, and emulsification) have been used in the food industry, some cases are inefficient in terms of the protection of microorganisms, therefore decreasing their viability. Some microcapsules, such as alginate-based capsules, possess porous networks that expose bacteria to the external medium, leading to unfavorable protection of probiotics in the gastric tract [265]. Therefore, alginate microcapsules without additional polymer coating could protect bacteria during bacterial storage, but lose protective function upon the exposure to acidic gastrointestinal conditions [215]. Additionally, the microcapsules' size is also one of the critical factors influencing bacteria protection. Heidebach and colleagues demonstrated that only capsules with a size between 0.2 mm and 3 mm could protect the encapsulated bacteria from the harsh gastrointestinal environments [24, 259]. Collectively, other methods should be developed to overcome these limitations.

To improve the performance of microcapsules produced by conventional approaches, one of the solutions is the direct coating on the bacterial or microcapsule's surface using different materials [242, 259]. These materials can interact with the surface, producing an attached layer on the bacteria or their microcapsules [259]. This coating layer can decrease the permeability of capsules, hence reduce the exposure of bacteria to air during storage, and promote their stability at low pH and high temperatures, thus improving their protective performance after oral administration [24, 264, 266]. Moreover, such bacterial surface coating allows live cells to inherit the new functions provided by the modified materials, such as adhesive properties or the capacity of controlled release of a micronutrient [267]. A vast range and combination of coating materials, including chitosan, PLL, alginate, starch, silica, gum, and gelatin, have been used, and various coating techniques, such as layer-by-layer, coacervation, and biomimetic mineralization, have been applied to produce these coatings on bacteria.

5.2.1. Layer-by-layer

Layer-by-layer is the continuous absorption of diverse materials to one surface, which is performed by subsequently assembling materials with the opposite surface charges. This technology relies on the chemical electrostatic interactions of positively and negatively charged materials [268]. Since microorganisms exhibit a negatively-charged surface due to the phospholipids and proteins on the cell membrane [269], multilayers of alternatively oppositely-charged materials can be coated on its surface *via* electrostatic binding. Thus, a coating can be created on the surface of bacteria to form a protective microcapsule. Anselmo *et al.* combined chitosan with alginate to encapsulate *Bacillus coagulans* using a layer-by-layer method (**Fig. 8**) [185]. In short, the cationic chitosan layer and anionic alginate layer were alternately coated onto bacteria *via* electrostatic attractions for up to three bilayers. This chitosan-based layer-by-layer technology improved the survival of encapsulated bacteria against acidic and bile salt conditions as well as enhanced the capacities of mucoadhesion and growth on intestinal tissues after oral delivery. Moreover, the layer-by-layer coating could also be applied on the surface of bacterial microcapsules (*e.g.*, alginate microcapsules) to further enhance their stability in stomatal and gastrointestinal environment and modulate the release of bacteria. For instance, Lin *et al.* designed alginate-chitosan-alginate (ACA) microcapsules to encapsulate bacteria through layer-by-layer technology, with alginate and chitosan cross-linked by ionic bonding on the alginate microcapsule surface [187]. Results showed that the ACA microcapsules exhibited superior mechanical and chemical stability in simulated gastrointestinal conditions. Similarly, a novel alginate-PLL-pectin-PLL-alginate (APPPA) microcapsule was formulated using layer-by-layer technology [235]. This type of multi-layer microcapsule presented excellent stability in simulated gastrointestinal conditions than alginate microcapsules.

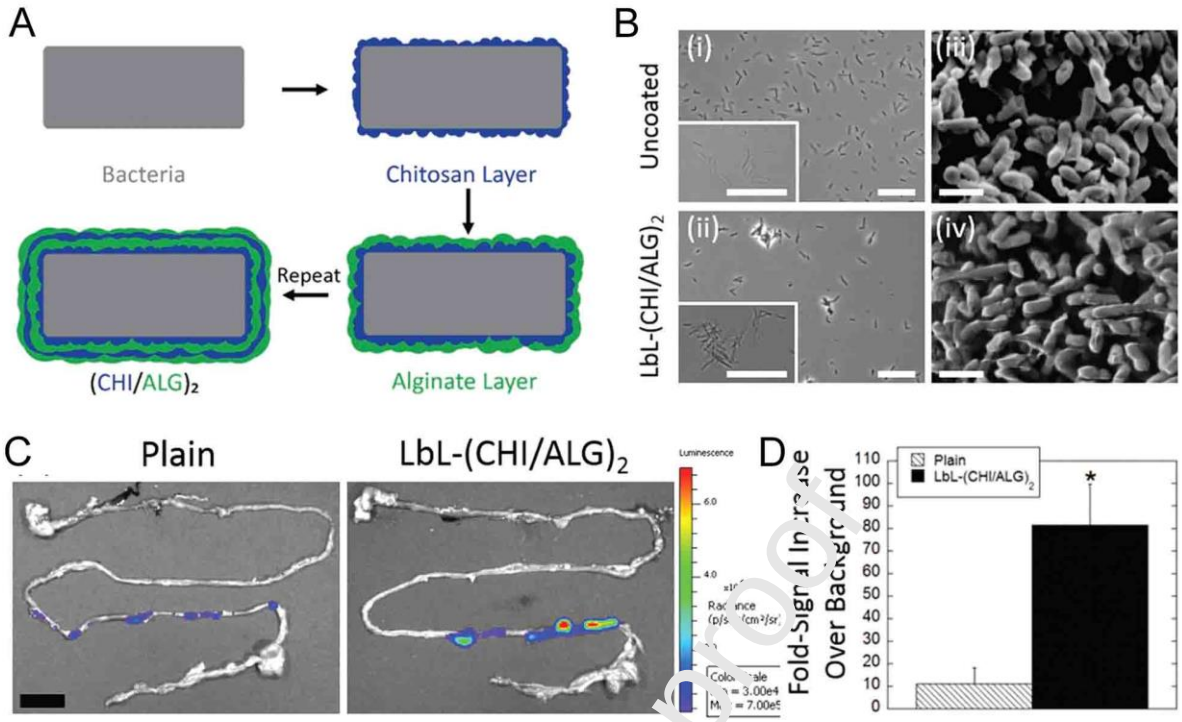

Fig. 8. Layer-by-layer (LbL) encapsulation of probiotics for oral delivery to intestine. (A) Schematic presentation of LbL template of chitosan and alginate on probiotics. (B) Bright-field and SEM images of uncoated-*Bacillus coagulans* (i, iii) and LbL-$(CHI/ALG)_2$-*Bacillus coagulans* (ii, iv). Scale bars of bright field and SEM are 25 μm and 2 μm respectively. (C, D) Typical IVIS images (C) and fold-signal increase (D) of plain-*Bacillus coagulans* and LbL-*Bacillus coagulans* 1 h after oral feeding. Scale bar: 1.5 cm. Reproduced with permission [185]. Copyright 2016, Wiley-VCH.

An interesting characteristic of this coating technique is the ability to control the thickness of layers. Cook *et al.* reported that the thickness of the chitosan-based coating layer on alginate microcapsules was increased with its immersing time. After 1-min immersion of alginate microcapsules (1 mm) in chitosan coating solutions, a coating layer with a minimum thickness of 8 μm was formed; while after 2400-min immersion, the thickness of the coating was 24 μm [219]. Several factors were identified to affect the adsorption of materials in the layer-by-layer process, including pH, ionic strength, temperature, adhesion time,

polyelectrolyte molecular weight, or the addition of external substances (proteins or surfactants) [243].

In conclusion, layer-by-layer is a low-cost, easy, efficient, and renewable method for the coating of bacteria, in mild conditions and aqueous solutions with natural charged materials. The main disadvantage of this method is the discontinuous process and the time-cost of the adhesion of each layer [243].

5.2.2. Coacervation

Coacervation is a procedure to isolate colloidal particles from a solution and deposit them around the core material. Three steps are containing in this method including phase separation, deposition, and solidification [244]. Firstly, the coating material comprising one or more polymer goes through a phase separation procedure to form a coacervate. Then, the coacervate nucleus rapidly adsorbs on the surface of emulsified core material owing to the decrease of surface area and total free interfacial energy in the system, producing a uniform coating layer on the core particles. Lastly, the coating layers are solidified by crosslinking using the chemical, thermal, or enzymatic method, followed by collecting the formed microparticles *via* filtration or centrifugation [244, 270]. Besides the application of coacervation in the entrapment of flavors, preservatives and enzymes [271], this technique has also been used for the microencapsulation of bacterial cells with high payloads [272-276]. For example, Eratte *et al.* co-encapsulated tuna oil and *L. casei* 431 in a single whey protein isolate (WPI)-gum Arabic (GA) complex coacervate microcapsule to enhance bacterial viability [276]. Hernández-Rodríguez *et al.* entrapped *L. plantarum* in whey protein isolate/κ-carrageenan complex coacervates [272]. The complex coacervates prepared by the combination of whey protein isolate and κ-carrageenan with a weight ratio of 16.7:1 exhibited a more effective resistance against simulated gastrointestinal conditions compared with that with ratios of 10.0:1 and 3.3:1, providing structural elements and carriers for orally-delivered

probiotics. However, the coacervation method has a major drawback of the difficulty in obtaining capsules with small sizes [277, 278]. Moreover, the complexity and the cost of the process also limit its application in a narrow range of bacteria [270].

5.2.3. Biomimetic Mineralization

Biomineralization is a process, in which living organisms employ organic substances to form inorganic mineral-based structures [245]. During the evolution of natural systems, a variety of biominerals, including bones, teeth, carapaces, shells, spicules, and so on, are developed by living organisms, exhibiting distinct structures and possessing considerable functions, including mechanical support, protection, mobility, and sensing of signals [246]. With the exploration of the mechanism of these natural mineralization processes, artificial design, and rational integration of mineralizable macromolecules can facilitate the modification of non-mineralized organisms. To date, a range of living organisms, such as bacteria, algae, yeast, viruses, and even human cells, have been favorably applied for biomineralized modification using a diverse variety of methods, constructing numerous novel living-mineral integrations with specific structures and unique properties [245, 267].

As one of the living-mineral strategies, coating a mineral on the external surface of bacteria through artificially-managed biomineralization process has become a novel approach for bacterial encapsulation [279-282]. Silica and calcium carbonate are two paradigms of inorganic bacterial encapsulation systems, which have been widely studied because of their low toxicity, thermal and mechanical stabilities, and excellent biocompatibility as well as biodegradability [283-286]. The presence of a shell on the surface indeed has a remarkable stabilizing effect on bacterial cells at considerably high temperatures, enabling the storage of bacteria at room temperatures in the air without any rigorous requirements. As a representative example, yeast spores and bacteria recently were immobilized in silica gel, in which their enzymatic activity was maintained [287-291]. Nassif *et al.* developed a technique

to entrap *E. coli* in a silica gel and confirmed the benefits of generating mineral shells to the survival of cells. The metabolic activity of encapsulating *E. coli* declined slowly, and half of cells could survive after a month [290]. Yeast cells (*S. cerevisiae*) wrapped in mineralized shells were still viable for a month in water, showing a higher survival level compared with untreated cells in the same condition (**Fig. 9**) [291]. Moreover, accommodation of bacterial cells in a porous mineral shell allows only molecules smaller than shell pores to pass through and arrive the interior, serving as a solid exoskeleton for protecting cells against external damage. Therefore, such a biomimetic mineralization approach has been employed to encapsulate bacteria or probiotic for protection against harsh environments, like gastrointestinal conditions. Haffner *et al.* encapsulated *Lactobacillus rhamnosus* (*L. rhamnosus*) *GG* in core-shell alginate-silica microcapsules by forming a silica shell on the electro-sprayed alginate ion gel core *via* hydrolysis/condensation of alkoxysilane precursors (**Fig. 10**) [190]. Because of the non-swelling feature and mesoporosity of silica shells, this mineral coating could prevent cell leakage as well as ensure bacterial proliferation inside the microcapsules, which directed an application in oral administration of probiotics.

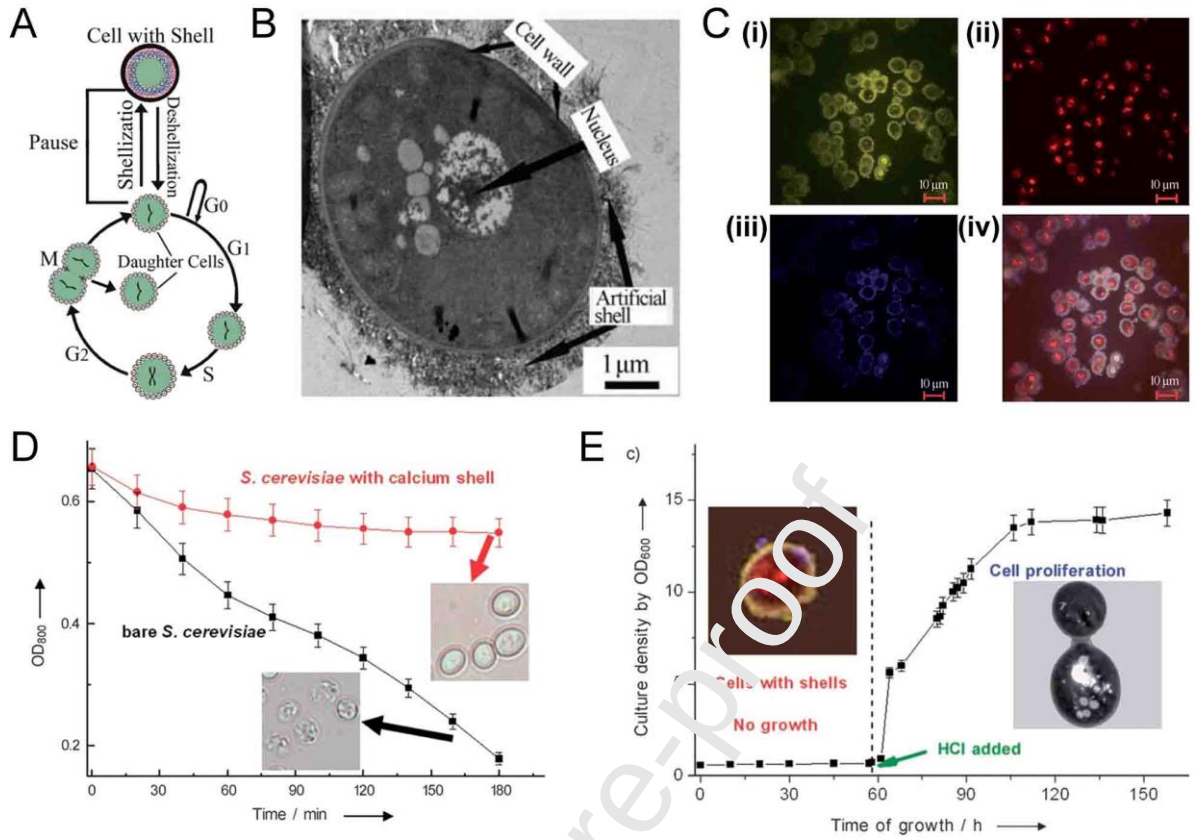

Fig. 9. Yeast cells biomineralized with an artificial calcium shell. (A) The schematic presentations of the lifecycle of normal and encapsulated *S. cerevisiae* cells. (B) Ultrathin section image of the surface-mineralized yeast cells. (C) Confocal microscopy images of the encapsulated *S. cerevisiae*. i). iii) Calcium shell and cell walls are dyed with tetracycline hydrochloride (i) and fluorescent blue (iii) respectively. ii) Red spots indicate the living cells. iv) Merged image. (D) Viability of *S. cerevisiae* in the presence of zymolyase. The insets are the relevant optical images of *S. cerevisiae* at 180 min. (E) Growth curve of *S. cerevisiae* with and without the calcium shell. 1 mM HCl was added at t = 60 h to trigger the disassembly of the mineral shell. Left inset is a fluorescence image suggesting the living cells; right inset is a TEM image indicating a separated cell. Reproduced with permission [291]. Copyright 2008, Wiley-VCH.

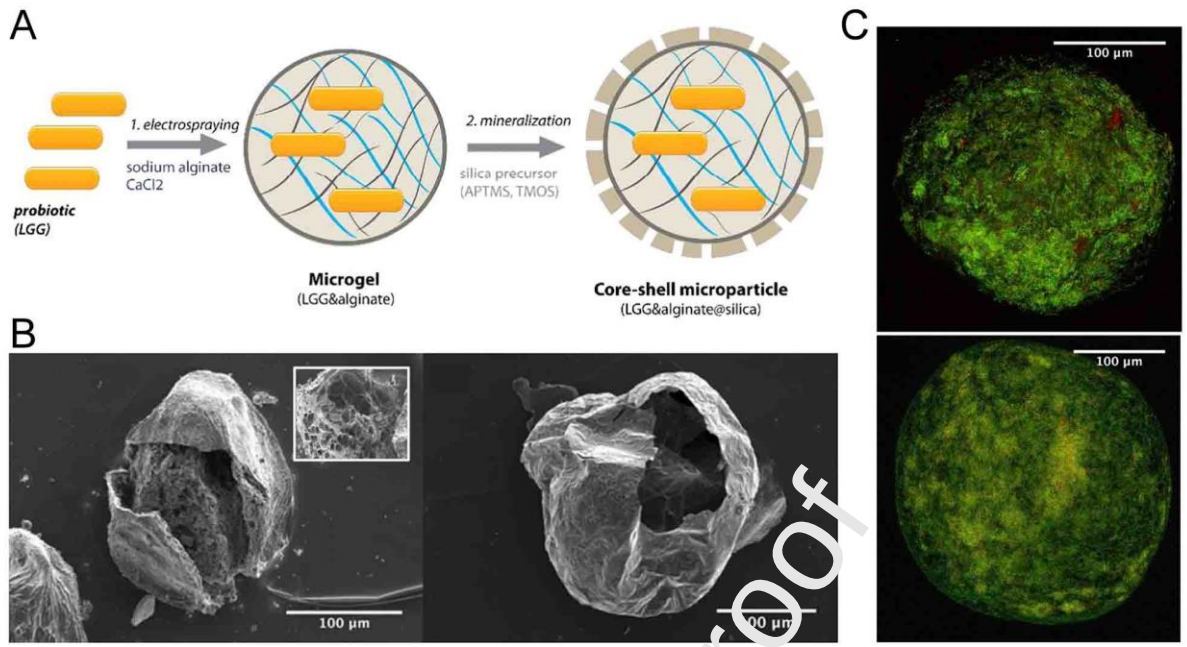

Fig. 10. Core-shell alginate@silica microparticles for probiotic encapsulation. (A) Graphical description of the encapsulation process. (B) SEM images of freeze-dried LGG&alginate and wet core-shell LGG&alginate@silica microparticles. (C) Confocal laser scanning microscope images of core-shell alginate@silica microparticles indicating the survival of entrapped LGG after 2 h immersion in simulated gastric fluid (up) and simulated intestinal fluid (down). LGG refers to *L. rhamnosus* GG. Reproduced with permission [190]. Copyright 2014, The Royal Society of Chemistry.

Compared with the polymer-based coating methods, the bacteria enclosed in biomimetic minerals like artificial shells exhibit much better stability and survivability. This technique protects bacteria from more harsh conditions, such as ultraviolet radiation and lytic enzyme. However, this mineralization-based surface coating method cannot be applied in all species due to their unfavorable structure and properties and sometimes affects the biological biosafety of bacteria. Moreover, its application in bulk encapsulation needs to be developed [245, 246].

5.2.4. Other Surface Coating Methods

Conventional surface-coating methods, that conjugate or absorb pre-formed polymers onto the cell surfaces, are constrained by low polymer grafting efficiency. To solve this problem, Niu *et al.* firstly reported the biocompatible controlled radical polymerization (CRP) technique, by which the surfaces of yeast or mammalian cells can directly initiate polymerization to achieve surface engineering [247]. This CRP offered a biocompatible technique with a unique advantage of surface-initiated polymerization that could form a network of functional polymers with high areal chain density. In another similar study, Magennis *et al*. employed bacterial redox systems to initiate copper-mediated radical polymerization (ATRP) on the bacterial cell surface, producing a layer of polymer *in situ* that was firmly bound to the microorganisms [248]. Further expansion of the bacterial redox chemistries to link fluorescent reporter molecules on polymers enabled the quick, easy, and synchronous binding and visualization of pathogens. Given that these methods can be easily extended to conjugate various functional polymers onto cell surfaces, these strategies exhibit great potential to be applied in bacterial encapsulation for oral delivery.

Overall, the final objective of surface-coating technology is to add materials onto the surfaces of bacteria cells to overcome challenges in the process of oral delivery, such as mechanical stability, chemical stability, biocompatibility, and permeability. Taking full advantage of the material's strengths while avoiding their flaws, surface-coating technology offers more selections of bacterial microencapsulation for oral delivery.

5.3. Microfluidics

Microfluidic is an increasingly powerful emulsification technique for microencapsulation and microcapsule fabrication [292, 293]. Different from the conventional emulsification methods, microfluidic technology generates microspheres through a microfluidic chip device, in which the internal and external phases are pumped into two inlets by two separate digital syringes that can accurately control the flow rates. In this way, the microfluidic technology

could produce monodisperse microspheres with accurately controllable dimensions in a replicable manner, while avoiding the undesirable batch-to-batch changeability and low encapsulation efficiency, serving as a promising tool for constructing micro/nano-drug delivery systems like liposomes, microgels, and polymer microspheres [294-297].

Recently, the emphasis of the application of microfluidic technology has been focused on the microencapsulation of living microorganisms using different devices and materials for their applications in cancer therapy, drug delivery, environment recovery, food industry, and cell culture contexts [249, 298-302]. Lee *et al.* encapsulated *E. coli* in polyethylene glycol diacrylate (PEGDA) micro-sized droplets *via* a microfluidic based chemical polymerization [303]. The encapsulated *E. coli* remained viable and efficiently expressed the fluorescent proteins inside the microbeads because of the unique features of microdroplets. Barlow *et al.* reported the microfluidic generation of biodegradable synthetic polymer microspheres containing *Bacillus subtilis* (*B. subtilis*) [304]. Using a double-emulsion microfluidic device equipped with glass capillaries, *B. subtilis* were completely entrapped in semi-permeable membranes containing poly(ethylene glycol)-*b*-poly(D,L-lactic acid) (PEG-PDLLA). The favorable permeability of this polymer membrane allowed sufficient bacterial proliferation, metabolite-inducible gene transcription, and rapid biofilm growth. These microfluidic-based techniques provided a high encapsulating efficiency per particle and a low consumption of reagents, thereby enabling the encapsulating process to be low-cost [249]. Moreover, this method could be easily performed in industrial-scale production by simply increasing the bacterial cell numbers, meeting the requirements of the pharmaceutical industry [292]. However, the feasibility of the application of microfluidic technology in the encapsulation of bacteria for oral delivery is still required to be explored by selecting the appropriate encapsulating materials.

6. Biomedical Applications of Oral Delivery of Bacteria

Recent advancements in the knowledge of the composition and metabolism of the human microorganisms have established their important influence on the development of human diseases [305, 306]. Specifically, the cumulative data indicate the crucial role of microorganisms in the etiology of gastrointestinal disorders or cancers by affecting inflammation, DNA damage and apoptosis [5]. Thus, beneficial bacteria have been used as a therapeutic to recover the balance of the metabolism of human microbiota to resist the advancement of these diseases [12]. Meanwhile, bacteria can also interact closely with their niches in the body, respond to various diseases, and be appropriately adjusted to detect and produce physiological levels of desirable biomolecules. Integration of these characteristics with their natural capacities (*e.g.* chemotaxis and biomolecule secretion) may permit engineering bacteria-based systems to outperform conventional diagnostics and therapeutics [15, 18]. Overall, these features make bacterial therapy one of the most promising approaches in disease management.

Among the various administration methods, the oral route is considered the most convenient approach. It is worth mentioning that recent studies have demonstrated the great application of gut microbiome in disease management through the regulation of gastrointestinal tract signal pathways [9, 10, 88, 305]. Thus, oral administration of bacteria is the most suitable and direct way to modulate the balance of the gut microbiome, facilitating the transplantation of bacterial products as the oral modality. Moreover, the integration of biomaterials and encapsulation technologies with bacterial therapy has also promised enhanced delivery efficiency and reduced side effects during the biomedical application of oral bacterial delivery. Thus, by carefully designing the therapeutic bacteria using genetic engineering and rationally selecting appropriate encapsulating material and technologies, oral delivery of novel bacteria has been extended to a broad scope of applications in biomedicine. In this section, we categorize and discuss various biomedical applications of bacteria *via* the oral route, including disease prevention, diagnosis, treatment, and others. **Table 4** summarizes

the multiple target organs and diseases, in which engineered bacterial therapeutics were applied.

Table 4. Biomedical applications of orally-delivered bacterial therapy.

	Bacteria	Disease	Mechanism	Reference
Diagnosis	*E. coli*	Inflammation	Genetic circuits to detect tetrathionate	[307]
	E. coli Nissle	Inflammation	Genetic circuits to detect *P. Aeruginosa*	[145]
	E. coli Nissle *1917*	Liver cancer metastases	Detect liver metastases in urine	[60]
prevention	*L. Lactis*	Anti-*H. pylori* vaccines	Pylori lipoprotein Lpp20 (genetic circuits)	[308]
	B. subtilis spores	Tuberculosis vaccines	*H. pylori* urease B protein	[309]
	B. subtilis spores	Tuberculosis vaccines	Mycobacterium tuberculosis	[310]
	L. lactis	Multidrug resistant *Enterococcus faecium*	Express antimicrobial peptides	[311]
	L. Lactis	Diarrhea	Express TcdA and TcdB (genetic circuits)	[312]
Gastrointestinal diseases	*L. lactis* (LL-Thy12)	Crohn's disease	Thymidylate synthase replaced with human interleukin-10	[313]
	B. longum	Ulcerative colitis	Express α-melanocyte-stimulating hormone	[314]
	L. lactis	Colitis	Express immunosuppressive IL-27	[35]
	Lcr35 and LaBi	Diarrhea	Secrete proinflammatory cytokines	[315]
Cancer	*E. coli*	Cancer	Sense glucose and ribose sugar	[115]
	B. longum	Hepatic metastasis from a solid tumor cancer	Engineered bacteria carrying endostatin gene	[316]
	B. longum	Cancer suicide therapy	HSV-TK and GCV	[317, 318]
	Salmonella	Cancer	Induce tumor cell apoptosis	[63, 64]
	Salmonella	Anti-tumor immune response	$CD4^+$ and $CD8^+$ T cells	[319]
	E. coli	Radiotherapy of cancer	Generate ClyA	[157]
Diabetes	*L. lactis*	Type 1 diabetes	Express GAD-65 and IL-10	[142]
	L. lactis NZ9000	Type 1 diabetes	Express protein HSP65-6P277	[320]
	L. lactis NZ3900	Type 2 diabetes	SCI-59 displayed onto the surface of NVBs	[321]
	B. longum	Type 2 diabetes	Glucagon-like peptide-1 (GLP-1)	[322]
	L. gasseri ATCC 33323	Type 2 diabetes	Glucagon-like peptide-1 (GLP-1)	[37]
	L. paracasei	Type 2 diabetes	Exendin-4 peptide, a GLP-1 receptor agonist	[323]

6.1. Disease Prevention

Bacteria can be designed as a prophylactic system expressing antigens of specific pathogens, thereby triggering the body's immune responses and achieving disease prevention. For example, *L. lactis* could be engineered to express the *Helicobacter pylori* (*H. pylori*) lipoprotein Lpp20, which could be used as vaccines against *H. pylori* [308]. Similarly, *B. subtilis* spores were genetically designed to express *H. pylori* urease B protein and the antigens of *Mycobacterium tuberculosis* respectively, serving as vaccination against tuberculosis [309, 310]. Furthermore, most asthmatic patients were allergic to house dust mites (HDM), and most HDM-allergic patients showed an active reaction to Der p2, a type of HDM allergen. Feeding mice with recombinant Der p2-expressing *L. lactis* could deter the inflammation by reducing IgE antibody secretion and T cell response upon HDM exposure [324].

Additionally, bacteria have also been used to prevent drug resistance. The extensive use of antibiotics increased the resistance of pathogenic bacteria. This risk of antibiotic resistance could be reduced by engineered probiotics through controlling the release rate of antimicrobial agents. For example, Kathryn *et al.* engineered *L. lactis* to detect the pheromone cCF10 of *Enterococcus. Faecalis* (*E. faecalis*) and kill the multidrug-resistant *E. faecalis* by secreting bacteriocins [311]. Therefore, engineered bacteria could effectively impede the growth of drug-resistant microbes.

6.2. Disease Diagnosis

Bacterial sensing circuits are capable of being designed to sense molecules that are related to disease development, such as cytokines, hormones, physiological stimuli, and metabolites [126, 307, 325-329]. Based on this function, bacteria are able to be engineered as diagnostics for responding and reporting human diseases in the body. Engineering bacteria to detect short-lived molecules, which could not be readily captured and quantified by conventional noninvasive test methods because of the degradation, modification and absorption before

exiting the intestine, is a perspective approach to measure unique biomarkers [15]. Moreover, diagnostic bacteria are also able to be armed with additional functions, like recording the measurements and delivering therapeutics, indicating the future potential of this strategy.

Recently, probiotic strains are engineered to detect gut inflammation by sensing their metabolites using a gene circuit. Riglar *et al.* recently constructed a memory circuit in commensal murine *E. coli* to sense and record the exposure to tetrathionate that was produced during gut inflammation [307]. They demonstrated the feasibility of this engineered bacteria noninvasively reporting transient molecules *in vivo* by fecal testing, which enabled the observation of mouse intestinal inflammation for more than 6 months. This durable performance allowed the sustained monitoring of the inflammatory condition until the initial tetrathionate signal was disappeared. Daeffler *et al* equipped *E. coli* Nissle 1917 with the sensors for the detection of the increase in thiosulfate in the mouse model of chemically-induced colitis [325]. After oral feeding, the results from flow cytometry analysis of colon and fecal samples demonstrated that the thiosulfate sensors in mice were activated by colon inflammation, indicating these engineered bacteria could be applied for diagnostics.

Owing to the ability to selectively home to specific sites like tumors, bacteria were also engineered to act as a sensitive diagnostic tool for detecting and reporting on the presence of cancers. Danino *et al.* developed a synthetic diagnostic tool, an orally administrated PROP-Z platform where an *E. coli* Nissle 1917 strain was equipped with a special gene circuit to detect liver metastases through signals in urine (**Fig. 11**) [60]. Such *E. coli* Nissle 1917 was able to pass through the gastrointestinal environment and preferentially colonize in hepatic metastases, expressing high levels of lacZ in the lesion-developed tissue. Then, lacZ metabolized LuGal, a soluble conjugate of luciferin and galactose injected in mice, into luciferin, which eventually could be examined in the urine. Using this method, an average signal-to-noise ratio of luciferase produced by PROP-Z-colonized liver metastases was measured to be about 3.6 in contrast to that in healthy animals, achieving noticeable urinary

color changes in the test cases. Although the transplantation of *E. coli* passing through the intestines was examined in the human body, it was only observed in few healthy individuals and could not be applied for malignant masses smaller than half a centimeter in diameter, limiting the application of *E. coli* as a systemic diagnostic. To overcome this limitation, Panteli *et al.* engineered attenuated *S. typhimurium*, preferentially accumulating in tumors and microscale metastases as small as five cell layers thick, to express a fluorescent molecule, ZsGreen, for reporting tumor. Based on the testing on the tumor-on-a-chip device, the measured rate of ZsGreen release could achieve the detection of 0.043 mm^3 tumor masses, which was 2600-fold smaller than the current limit of tomographic techniques [329].

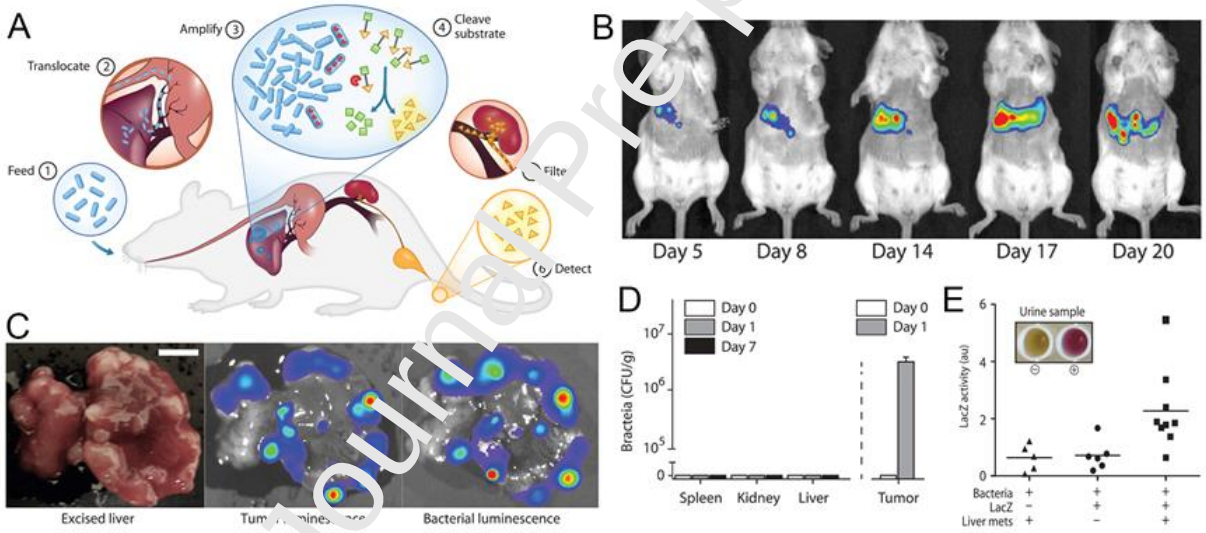

Fig. 11. Programmable probiotics for the detection of cancer *via* the analysis of urine. (A) The PROP-Z diagnostic platform, consisting of *E. coli* Nissle 1917 transferred with a lacZ vector and a genomically-integrated *luxCDABE* cassette, for noninvasive cancer detection. After oral gavage, the PROP-Z rapidly enter the mouse gastrointestinal tract and metastatic tumors in the liver, followed by expressing enzyme lacZ (green) to cleave injected LuGal into substrates (red and yellow). The substrate (yellow) was filtered through the renal system, and mice urine was used for detection. (B) The growth of metastases was monitored by IVIS after the intraperitoneal injection of D-luciferin. (C) Photo of excised livers from oral PROP-Luc mice

at 24 hours (left), bacterial luminescence (right), and active luminescence of tumors was soaked in luciferin (middle). (D) Traditional colony counting of PROP-Z remaining in healthy mice (left) or liver metastases-bearing mice (right). (E) PROP-Z activity detected by injection of LuGal to produce luminescent luciferin in the urine [60]. Reproduced with permission. Copyright 2015, Science Translational Medicine.

The applications of bacteria in disease diagnosis, especially for early detection of cancers, may extend the survival time of patients. Compared to biopsy, the traditional tumor diagnostic approaches, oral bacterial delivery can alleviate the patients' pain more effectively [15, 330].

6.3. Disease treatment

6.3.1. Gastrointestinal diseases

Gut microbiota, containing a vast diversity of microorganisms, reach extraordinary densities and communicate with the intestinal mucosa to affect intestinal permeability, which is essential for the absorption, distribution, metabolism, and excretion of nutrients [7]. The gut microbiota has been regarded as a potent and selective strength for maintaining the homeostasis of hosts. Disrupting this homeostatic host-microbe interaction will facilitate the development of diseases such as inflammatory bowel diseases like ulcerative colitis and Crohn's disease [331, 332]. Long-term inflammation increases the risk of colorectal cancer (CRC) [166]. Therefore, the restoration of gut microbiota symbiosis and composition is crucial to the prevention and treatment of gastrointestinal dysbiosis-relative diseases [333-335].

Fecal microbiota transplantation (FMT) is a potential method to restore the diversity of intestinal bacteria and reduce inflammation, which is achieved by orally transferring fecal microbiota harvested from normal human to patients [336, 337]. Recently, a number of clinical trials have focused on the use of FMT to treat various disorders, including irritable

bowel syndrome, inflammatory bowel diseases, *Clostridium difficile* (*C. difficile*) infection, insulin resistance, multiple sclerosis, and idiopathic thrombocytopenic purpura [338-341]. Among them, the most successful case of FMT is the treatment for recurrent intestinal infection and diarrhea caused by *C. difficile*, showing a high efficiency with a success rate of 90% [337]. However, the efficacy of this therapy for treating other gastrointestinal disorders remains suboptimal, and the mechanism of natural and introduced microbial strains is still largely unknown [336].

Another method to alter the intestinal microbiota for the treatment of gastrointestinal disorder is the engineering of the orally-delivered microorganisms for expressing functional or therapeutic molecules. Some engineered microorganisms have been demonstrated with potentially prophylactic and therapeutic activities against gut infections and inflammation. One high-profile example was engineering orally-delivered *L. lactis* to secret IL-10, which were used to treat colitis with a 50% reduction of inflammation *in vivo* [34]. To obtain an enhanced efficiency, *L. lactis* strains were engineered to co-express some other anti-inflammatory cytokines, including interleukin-27 (IL-27), thymic stromal lymphopoietin (TSLP), and anti-TNF (tumor necrosis factor), for a synergetic effect of colitis treatment [35, 36, 342]. Such engineered *L. lactis*-based therapy increased the mucosal bioavailability of these cytokines at the site of inflammation in the intestine with safety and good tolerance [343]. Besides the secretion of soluble therapeutic proteins, microbes have also been designed to express therapeutic molecules for mucosal-healing promotion, which is essential to combat the effects of inflammatory bowel diseases, fistulae, and ulcers. Praveschotinunt *et al.* presented an engineered orally-delivered *E. coli* that secreted the monomer unit of curli fibers (CsgA) to self-assemble extracellularly into a multivalent material decorated with trefoil factors (TFFs)-displaying domains for inflammatory bowel disease treatment [344]. These displayed domains could bind to the mucosal layer of the epithelium, formulating fibrous hybrid matrices to promote intestinal epithelial integrity *in situ* (**Fig. 12**). This probiotic-

associated therapeutic curli hybrids (PATCH) could ameliorate colitis inflammation triggered by dextran sodium sulfate (DSS) in mice.

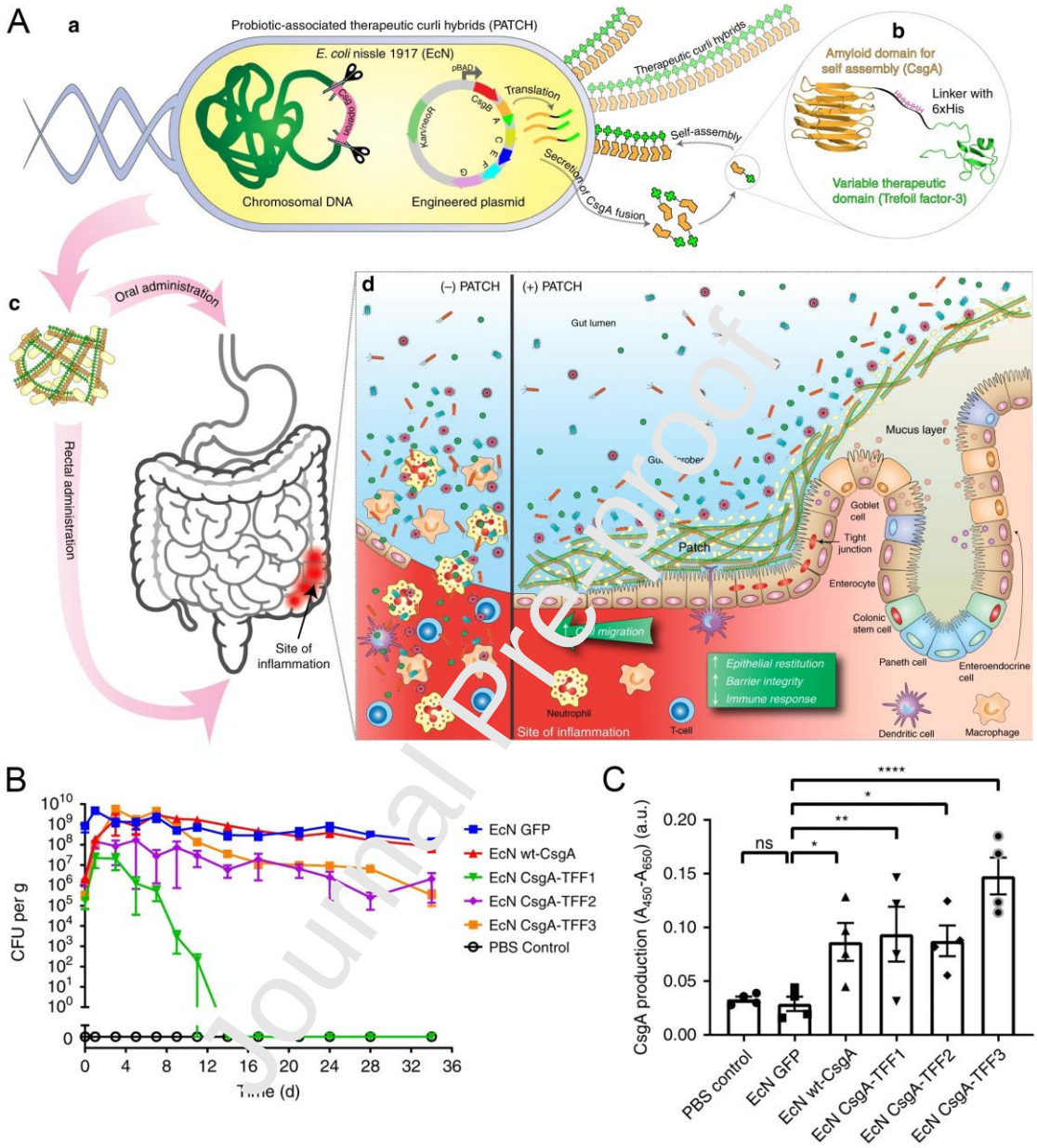

Fig. 12. Intestinal delivery of matrix-tethered therapeutic domains using orally-administrated engineered *E. coli* Nissle 1917 for inflammatory bowel disease treatment. (A) The preparation mechanism and effects of probiotic-associated therapeutic curli hybrids (PATCH). Genetically engineered *E. coli* can secrete chimeric CsgA proteins (b) encoded by curli operon (a). After oral administration (c), programmed *E. coli* interacts with the colonic mucosa. Produced PATCH can reinforce barrier function, promote epithelial reconstruction, and inhibit inflammatory signals to alleviate inflammatory bowel disease activity (d). (B) The

retention time of engineered *E. coli* in the mouse gut. (C) CsgA production from the fecal sample. Reproduced with permission [344]. Copyright 2019, Nature Communications.

In addition, chemotherapeutic agents, such as 5-fluorouracil (5-FU), may induce intestinal disorders like mucositis [10]. Recent studies showed that probiotics could been used to ameliorate these pervasive side effects caused by chemotherapy [315, 345-347]. As an example, Yeung *et al.* verified that probiotics (Lcr35 and LaBi) could alleviate this 5-FU-mediated mucositis in the mouse model [315]. After oral administration of *L. casei rhamnosus* and *B. bifidum* to the mice with 5-FU-induced mucositis, the symptoms of diarrhea were significantly alleviated with a significant reduction of diarrhea scores from 2.64 to 1.45 and 0.80, respectively. The damage in jejunal villi caused by 5-FU could also be repaired. This elimination of 5-FU-induced side effects resulted from the inhibition of the expression of proinflammatory cytokines by these probiotics. Bowen *et al.* discovered that VSL#3, containing a mixture of *Streptococcus thermophiles* (*S. thermophiles*), *B. breve*, *Lactobacillus paracasei* (*L. paracasei*), *B. infantis*, *B. longum*, *Lactobacillus delbrueckii subsp. bulgaricus*, *L. acidophilus*, and *L. plantarum*, alleviated diarrhea and weight loss in rats treated with irinotecan, accompanied by increased intestinal crypt hyperplasia and apoptosis inhibition [347]. Collectively, the oral administration of probiotics has become an alternative strategy for the prevention or treatment of chemotherapy-induced mucositis.

6.3.2. Cancer

Although cancer is usually regarded as a disease caused by the host's genetics and environmental factors, microbes have been demonstrated with a close relationship with cancers, even contributing to about 20% of human malignancies [8, 348]. For instance, microorganisms residing in mucosal sites may turn into a portion of the tumor microenvironment of aerodigestive tract malignancies, while the intratumoral microorganisms

are capable of influencing the development and propagation of cancer in various ways [2, 166]. Moreover, some pathogenic bacteria inherently own a specific capacity of accumulating in tumors [13]. Several non-severely pathogenic bacteria can spread under the direction of the applied external forces, achieving controllable movement and cargo transport [92]. These features of bacteria have interrogated the role of bacteria in cancer therapeutics with a holistic perspective [3, 173, 349, 350]. The availability of bacteria-based anti-cancer therapeutics has gained attraction for over 100 years, from the Coley's toxins to the current synthetic biology-designed microbes and microbiota transplants [164]. Based on these mechanisms, the therapeutic effects of orally-delivered engineered bacteria for cancer treatment have been demonstrated in numerous experiments and introduced in the following section.

Firstly, owing to the inherent chemotaxis, magnetotaxis, and intestine-colonizing properties, some bacteria exhibit a high performance of accumulation in specific sites, allowing them to be used as an ideal vesicle for delivering therapeutics (*e.g.*, small molecular drugs, proteins, DNA) to tumors [16]. These therapeutics are always loaded on the surface of bacteria *via* physical attachments, chemical propagation, or biological reconstruction, formulating bacterial hybrids [93, 351-353]. Under the direction of bacteria, the anti-tumor drugs are preferentially delivered to the tumor sites. Park *et al.* used the layer-by-layer technique to fabricate polyelectrolyte multilayer (PEM) microparticles for loading doxorubicin (DOX) and magnetic nanoparticles (Fe_3O_4), followed by attaching these PEM microparticles to *E. coli* surfaces to form micro-swimmers (**Fig. 13**) [354]. These micro-swimmers could efficiently deliver the anti-cancer drug molecules enclosed in PEM microparticles to target breast cancer cells under the guidance of both chemotaxes (along with α-methyl-*DL*-aspartate) and magnetic field *in vitro*. In another similar study, Zhang and co-workers prepared magnetite nanostructured porous hollow microhelices by depositing the metal precursors (Fe^{2+} and Fe^{3+}) on *Spirulina platensis* (*S. platensis*), followed by succedent annealing and particle reduction [355]. The resulting biohybrids of superparamagnetic

microhelices possessed fascinating swimming characteristics when exposing to a rotary magnetic field, endowing them with a dramatic function of precise tumor localization. The author investigated the delivery performance of the stable cargoes (Au nanorods and RhB dye) using nanohybrids and further designed a Fe_3O_4-*S. platensis* biohybrid for tumor visualization [356]. These studies demonstrated the feasibilities of the delivery of anti-tumor reagents using bacterial nanohybrids. However, their anti-tumor efficiency was not explored *in vivo*. In a further study, Hu *et al.* developed a novel oral DNA vaccination encoding autologous vascular endothelial growth factor receptor 2 (VEGFR2), which was prepared by decorating attenuated *Salmonella* with synthetic nanoparticles self-assembled from cationic polymers and plasmid DNA (**Fig. 14**). [357]. The nanoparticle layers allowed bacteria to effectively evade from phagosomes, remarkably improved their acid resistance, and significantly promoted their spread into the blood circulation. After oral delivery of this vaccine, the successful suppression of tumor growth in tumor-bearing mice was achieved due to the synergetic effect of angiogenesis inhibition and tumor necrosis. In addition, Fan *et al.* decorated bio-mineralized gold nanoparticles (AuNPs) on the surface of *E. coli* MG1655, which was genetically encoded with the expression of therapeutic protein TNF-α under a thermally-sensitive promoter, to build a thermally-sensitive therapeutic system (TPB@Au) (**Fig. 15**) [358]. These transformed *E.coli* could targetedly colonize tumor regions, delivering the AuNPs to the tumor sites. After irradiation with near-infrared light, the AuNPs-generated heat would trigger the expression of TNF-α by thermally-sensitive bacteria, inducing apoptotic cell death in the tumor. The anti-tumor efficacy of this bacteria-nanoparticle integration was verified in both *in vitro* and *in vivo* studies. What's more, Song *et al.* presented an oral autonomous nanoparticle generator formed by modifying deoxycholic acid (DA) and loading doxorubicin (DOX) and sorafenib (SOR) on the spores of *Bacillus cagulans* (**Fig. 16**) [359]. The modified spores could efficaciously transport the drugs to cross the harsh acidic stomachic environment and release them in the intestinal environment, followed by

assembling to DOX/SOR/Spore-DA nanoparticles under the action of dissociated hydrophobic protein and the hydrophilic DA. The generating nanoparticles efficaciously penetrated the epithelial cells through the bile acid pathway, enhancing the basolateral release of drugs. Treatment of tumor-bearing mice with DOX/SOR/Spore-DA *via* oral administration led to significant inhibition of tumor growth after 14 days, showing superior therapeutic efficacy. Together, these paradigms demonstrate the modality of bacterial nanohybrids can guide the penetration and accumulation of delivered cargoes to a specific site to promote their therapeutic efficiency as well as reduce adverse effects, providing a novel carrier for multimodal anti-tumor therapeutics.

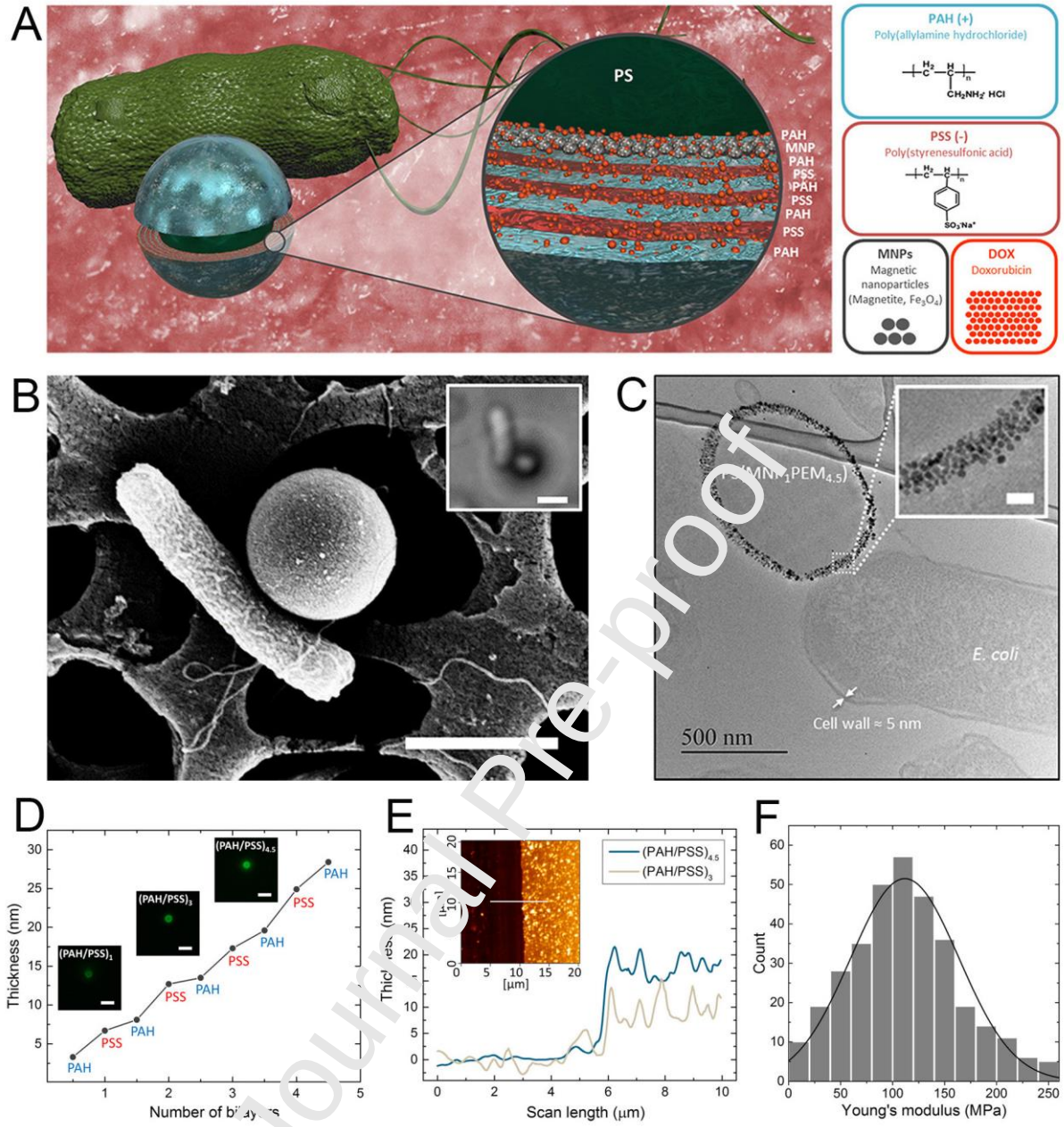

Fig. 13. Multifunctional bacteria-driven microswimmers for targeted drug delivery. (A) The design of multifunctional bacteria-driven micro-swimmer. The micro-swimmer was fabricated by attaching polyelectrolyte multilayer (PEM) microparticles, constructed by the layer-by-layer coating of positively-charged poly(allylamine hydrochloride) (PAH) and negatively-charged poly(sodium 4-styrenesulfonate) (PSS) to encapsulate DOX and magnetite nanoparticles (MNPs), on the *E. coli* surface. (B) SEM and optical image (the inset) of $PS(MNP_1PAH/PSS)_4PAH$-attached *E. coli*. Scale bar: 1 μm. (C) TEM images of the PEM microparticles. (D) The thickness of layers adsorbing PAH and PSS alternatively measured by

QCM-D analysis. Scale bar: 2 μm. (E) Thickness and surface profile of a $(PAH/PSS)_4PAH$ film detected using AFM. (F) Young's modulus distribution of $(PAH/PSS)_{20}PAH$ film detected by AFM nanoindentation. Reproduced with permission [354]. Copyright 2017, American Chemical Society.

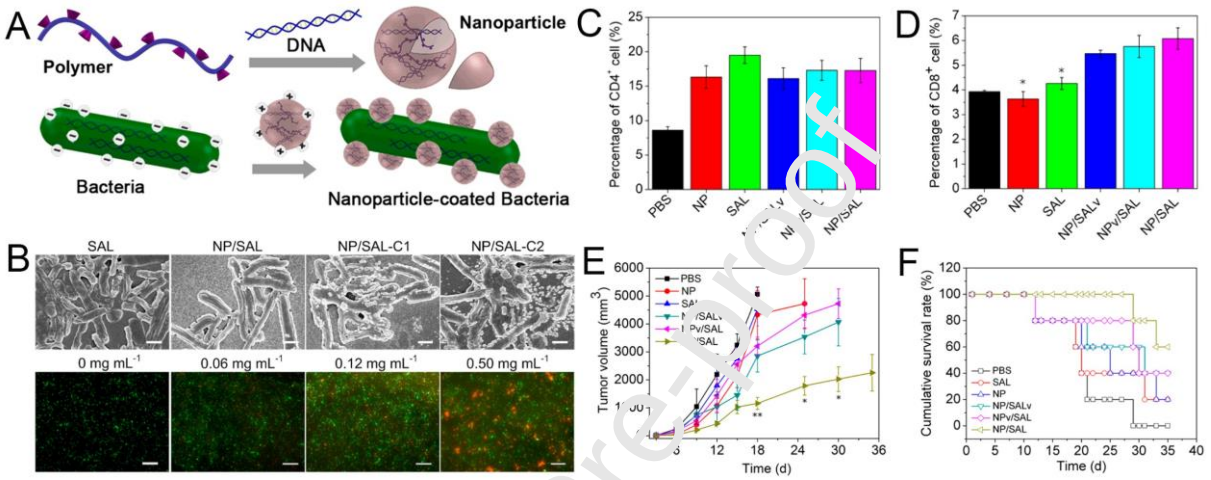

Fig. 14. Engineering nanoparticle-modified bacteria as oral DNA vaccines for cancer immunotherapy. (A) The preparation of the oral DNA vaccination encoding autologous vascular endothelial VEGFR2(NP/SAL). (B) Morphology and fluorescence microscopic images of naked *Salmonella* and *Salmonella* coated with different concentrations of polyplex nanoparticles. Green fluorescence represents naked bacteria and red indicates damaged cell membrane. (C, D) The percentages of $CD4^+$ (C) and $CD8^+$ (D) T cells after treatments with DNA vaccination. (E, F) The changes in tumor size (E) and survival rates (F) of tumor-bearing mice after different treatments. Reproduced with permission [357]. Copyright 2015, American Chemical Society.

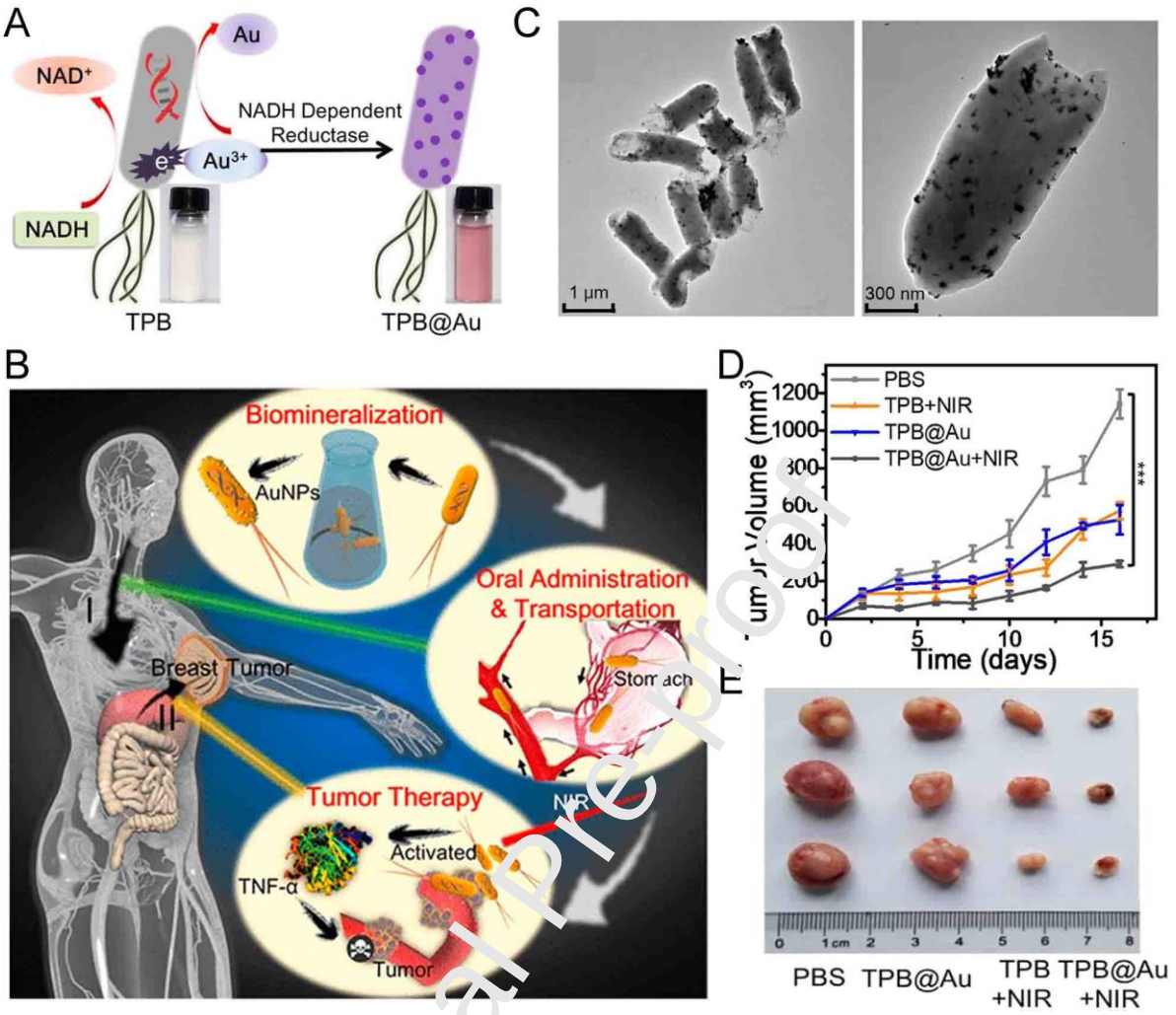

Fig. 15. Orally-delivered bacteria-mediated cancer therapy *via* photothermally-induced TNF-α expression. (A) Preparation of TPB@Au using enzymatic reduction. *E. coli* MG1655 was transferred with a customized plasmid pBV220, comprising a thermally-responsive promoter and a gene encoding TNF-α, followed by decoration with biomineralized photothermic gold nanoparticles on the bacterial surface to obtain TPB@Au. (B) Mechanism and therapeutic effects of TPB@Au. After oral administration, TPB@Au could reside into the gastrointestinal tract and be transported to internal microcirculation, targetedly delivering Au nanoparticles to hypoxic tumor regions, accompanied with the transcription of pBV220. With the irradiation of tumor by near-infrared light, AuNPs-generating heat induced the TNF-α expression, triggering apoptotic cell death. (C) TEM images of TPB@Au. (D, E) Tumor volumes (D) and representative photographs (E) after different treatments. Reproduced with permission [358]. Copyright 2018, American Chemical Society.

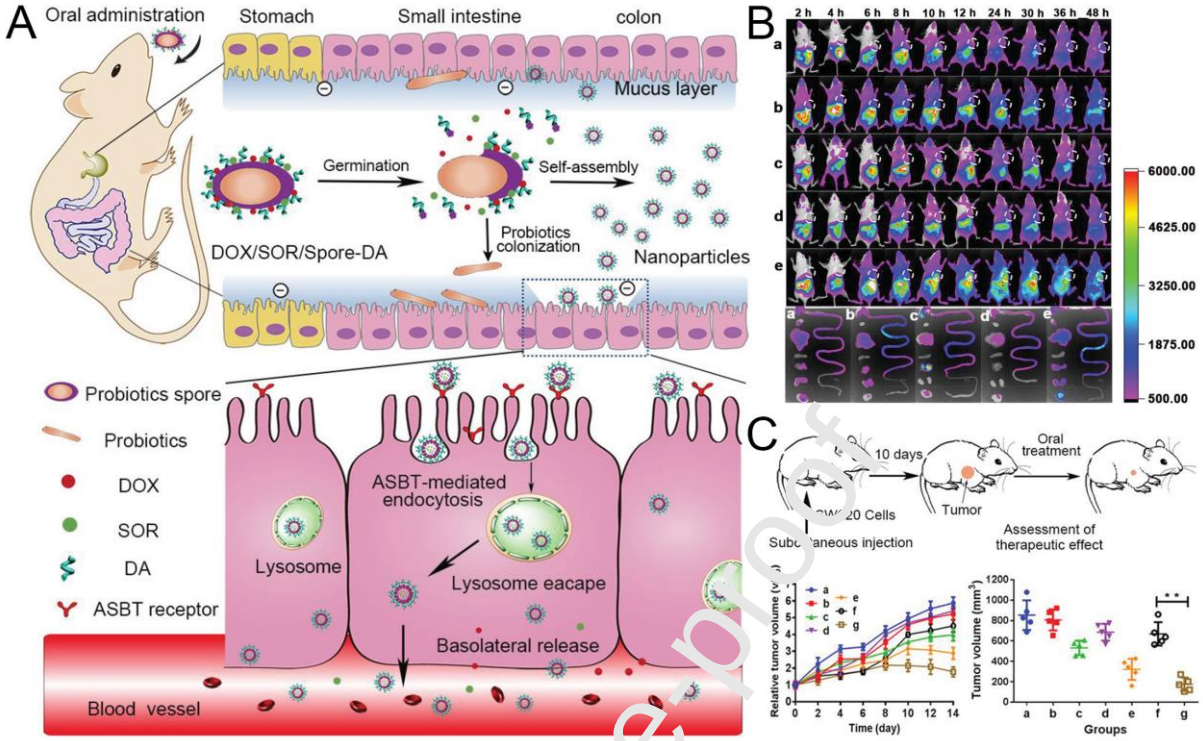

Fig. 16. A probiotic spore-based oral autonomous nanoparticles generator for cancer therapy. (A) Oral administration and transepithelial transport mechanism of DOX/SOR/Spore-DA. The surface of spores was decorated with deoxycholic acid (DA) to enhance absorption efficiency of the subsequent generating nanoparticles by intestinal epithelial to increase drug bioavailability. (B) The *in vivo* drug distribution of SW620 tumor-bearing mice after oral administration of IR780-labeled formulations. a. Free IR780, b. IR780/Spore, c. IR780/Spore-HA, d. IR780/Spore-DA + TCA, e. IR780/Spore-DA. (C) The average relative tumor volumes (left) and the real tumor volume (right) respectively after treatment with: a. saline, b. spores, c. DOX + SOR, d. DOX/SOR/Spore, e. DOX/SOR/Spore-HA, f. DOX/SOR/Spore-DA + TCA, g. DOX/SOR/Spore-DA. Reproduced with permission [359]. Copyright 2019, WILEY-VCH.

Meanwhile, bacteria can directly secrete cytotoxic agents *via* their native or inserted genes to destruct DNA or destabilize the cell membrane, allowing the bacteria to act as therapeutics for tumor elimination [13]. For example, Ryan *et al.* designed attenuated *Salmonella* to

express HlyE, a pore-forming cytolysin, to kill tumor cells [158]. To enable the bacteria to preferentially colonize the poorly-vascularized and hypoxic regions of tumors, the HlyE was carried under a novel and highly hypoxia-inducible promoter (FF+20*). After oral administration, the engineered *Salmonella* rapidly migrated into, colonized, and expressed HlyE in hypoxic regions of murine mammary tumors without damaging healthy host cells, realizing safe and effective bacteria-based cancer therapies. Besides bacterial toxins, three types of cytotoxic proteins, including FAS ligand (FASL), TNF-related apoptosis-inducing ligand (TRAIL), and TNFα, were also introduced into bacteria using genetic engineering to induce cancer cell apoptosis through death receptor pathways [123, 133, 134]. These therapeutic proteins were cytotoxic to the lung, ovarian, bladder, prostate, renal, colon, breast, glioma, and pancreatic tumors [360]. Bacterial delivery of these therapeutic proteins *via* oral route could overcome the drawbacks of systemic administration, such as hepatotoxicity and short circulatory half-life, enabling the production of proteins in tumor sites as well as maintaining a higher continual concentration.

More importantly, it is clear that microbes, particularly the gut microbiota, are capable of modulating the response of immune cells to fight against cancer cells [168-170, 305, 335]. Clinically, engineered *L. monocytogenes* is the most often used bacteria to induce potent T cell immunity for cancer suppression since it specifically infects professional APCs [53]. Oral administration of *L. monocytogenes* is the optimal route as the infection can originate at the mucosa [361]. Engineering *L. monocytogenes* to express cancer-specific antigens can trigger both innate and adaptive immunity against tumors [361]. Attenuated *L. monocytogenes*-based vaccines expressing HER2, prostate-specific antigen (PSA), the human papilloma virus (HPV) serotype 16 E7 oncoprotein or human mesothelin (CRS-207) have been tested in clinical trials [15]. Besides the stimulation of immune response by their intrinsic properties, bacteria can also stimulate immune cells to eliminate tumors by secreting specific immunomodulatory cytokines that have anti-tumor effects using genetic engineering [72, 362-

364]. Oral administration of *Salmonella* expressing IL-2 exhibited a prophylactical function to prevent tumor formation [179]. Similarly, treatment with *Salmonella* strains expressing IL-18, CCL21, and LIGHT (also known as TNFSF14) also induced the infiltration of leukocyte and neutrophil for tumor suppression [71, 73, 74]. These therapies had a good biocontainment and safety profiles, increasing the opportunities of inducing a robust immune response and successfully reversing tumor growth. Furthermore, recent studies have emphasized the contribution of the gut microbiome on the efficacy of modern cancer immunotherapy [12, 170, 305, 334, 335]. Immune checkpoint inhibitors, which block CTLA-4 expressed on activated effector T cells and programmed cell death protein 1 (PD1) or its ligand PD1-ligand 1 (PD-L1) to drive the patient's immune responses towards tumors, have demonstrated high efficiency in treating Hodgkin lymphoma, lung cancer, kidney cancer, melanomas, and bladder cancer [3]. Some studies have revealed that gut microbes play a crucial role in the immunostimulatory effects of CTLA-4 and PD-L1 blockade therapies [33, 76, 77]. The results showed that tumor-bearing mice with antibiotic treatment or free germ exhibited no response to CTLA-4 blockade therapy, but responded after feeding with *Bacteroides thetaiotaomicron* or *B. fragilis* [77]. Moreover, they also found that the combinational treatment with CTLA-4 blockade and *B. fragilis* on the tumor-bearing mice had a better effect than treatment with anti-CTLA-4 alone. Likewise, Sivan *et al.* found that *Bifidobacterium* could improve the anti-cancer effect of PD-L1 blockade therapy [76]. These results indicate the potential of bacteria as adjuvants for immune checkpoint blockade therapies. However, the mechanism of this combinational therapy is still unknown and a lot of efforts are required before the clinical trials.

Bacterial cancer therapy provides a new strategy that may be superior to traditional treatments of tumors. Based on the capacities of bacteria to act as carriers, secrete therapeutics and modulate the immune response, researchers can look for a more effective and targeted approach with fewer side effects to conquer cancers.

6.3.3. Diabetes

In addition to cancer, bacteria can also be used for the treatment of diabetes. Insulin plays an essential role in pharmacotherapy for diabetes. However, the subcutaneous injection of insulin causes much inconvenience to patients, while oral administration of insulin suffers from inevitable degradation by strong acids and various hydrolases in the gastric fluid [365]. Therefore, using engineered bacteria to deliver anti-diabetes therapeutics to induce the secretion of insulin from islet cells *in vivo* would be a promising method for diabetes treatment [9]. Robert *et al.* demonstrated that oral delivery of *L. lactis*, expressing T1D autoantigen glutamic acid decarboxylase (GAD)-65 and IL-10, had a significant effect on restoring normoglycemia as well as preventing the tolerance induction on NOD mice with type 1 diabetes (T1D) [142]. Mao *et al.* designed genetically-modified *L. lactis* NZ3900 to secrete single-chain insulin (SCI-59) analog, which was able to bind and stimulate insulin receptors expressed on 3T3-L1 adipocytes, followed by displaying SCI-59 on the surface of non-viable bacteria (NVBs) without genetic modification [321]. Orally-delivered SCI-59-NVBs might have significant therapeutic potential for treating diabetes mellitus, especially for T1D. Additionally, the hormone, glucagon-like peptide-1 (GLP-1), was engineered to be expressed by *B. longum* and *L. gasseri* to induce the differentiation of epithelial cells into glucose-sensing insulin-secreting cells, thereby improving the control of glucose in a rat model of diabetes mellitus [322]. In total, these results suggested the potential of engineered bacteria as delivery vectors for the treatment of diabetes *via* oral administration. Bacterial delivery is more effective in decreasing the level of glucose compared to the direct injection of these therapeutic molecules, which may be because of the shorter half-life of the peptide *in vivo*.

6.3.4. Obesity

Overweight and obesity refer to abnormal or excessive fat accumulation, which negatively affect human health. The obesity patients have doubled from 1980 to 2014, declaring that the worldwide epidemic of obesity is required to be taken under control. This obesity pandemic is closely associated with the disbalance between energy supplement and consumption [9, 366]. The recent development in next-generation sequencing technology and mechanistic testing in germ-free mice has confirmed the essential role of intestinal microbiota in body weight gain by affecting the whole-body metabolism and central food intake regulatory signals [306, 366, 367]. The evidence revealed that obesity was associated with the composition changes of the gut microbiota, and the gut microbiome in the obese mice appeared to be more effective in gaining energy from the diet [9]. Compared to lean mice, obese mice showed a reduced population of *Bacteroidetes* and a corresponding increase of *Firmicutes* [9, 368]. Obese humans also exhibited similar changes in intestinal microbiota with different ratios. Therefore, the relation between intestinal microbiota and obesity as well as the attempt to potentially develop new strategies for obesity prevention and treatment have attracted considerable research interest [153].

Probiotic bacteria have physiologic functions by affecting the gastrointestinal pathways and modulating the bacterial community in the intestines, contributing to the health of intestinal microbiota. Thus, oral administration of probiotic bacteria is an up-and-coming approach to restore the balance of the related gut microbiota for reducing the risk of obesity [11]. Preclinical evidence supports that the genus of *Lactobacillus* and *Bifidobacterium* strains are the main probiotics exhibiting the "anti-obesity" effects. Probiotic supplementation of high-fat and high-cholesterol mice with *Lactobacillus curvatus* (*L. curvatus*) HY7601 or *L. curvatus* HY7601 combined with *L. plantarum* KY1032 for nine weeks prevented body weight increase as well as decreased the weight of adipose tissue [369]. Similarly, supplement of high-fat diet (HFD)-feeding mice with *L. curvatus* HY7601 and *L. plantarum* KY1032 resulted in a 38% lower body weight gain compared with placebo and control groups [370].

Consistent with these observations, recent studies reported that a 12-week dietary supplementation of either *L. paracasei* CNCM I-4270, *L. rhamnosus* I-3690, or *Bifidobacterium animalis subsp. Lactis* I-2494 remarkably reduced HFD-induced weight gain, although the food intake of mice did not decrease [371]. Similar results were obtained in the research, where probiotics of *Bifidobacterium spp*. (*B. pseudocatenulatum* SPM 1204, *B. longum SPM* 1205, and *B. longum SPM* 1207 or *Bifidobacterium adolescentis* (*B. adolescentis*)) were provided to rats fed with HFD [372, 373]. In summary, these results provided hope for novel microbiota-based therapies for obesity in the future. However, these effects change dramatically, depending on both the type of bacterial strain and the host, requiring more studies to confirm.

6.4. Potential Applications

In recent years, significant efforts have been made to explore the relationship between gut bacteria and brain behavior [374]. The brain is nearly sterile due to the presence of a blood-brain barrier (BBB) that offers protection from blood insults like infections and maintains the homeostasis of neuronal environments [375-377]. However, there are still a few germs, such as *Neisseria meningitidis*, *Streptococcus pneumoniae*, *E. coli* k1, and group B *Streptococcus*, which can overcome the BBB, invading the meninges and inducing meningitis [376]. Researchers have presented some mechanism investigations of these brain-invading bacteria. For instance, due to slow blood flow, bacteria that may choose to adhere to the capillaries swim towards the subarachnoid space *via* glymphatic pathways. Additionally, pathogens inducing meningitis can attach a mucosal surface to cross the BBB, causing the infection of the cerebrospinal fluid (CSF) [376]. Thus, it seems possible that some other therapeutic bacteria can also cross the BBB to enter the brain, mimicking the invasion pathways of pathogens that cause meningitis.

According to these observations, gut bacteria have been explored to modulate the behaviors of the brain. A recent study by Olson *et al.* implied that the ketogenic diet (KD) was an effective treatment for refractory epilepsy [378]. Such a low-carbohydrate and high-fat ketogenic diet critically shaped the species composition and function of the gut bacteria, which was renewable and durable [379]. Olson *et al.* demonstrated that the KD altered the gut microbiotas in mouse models, and the gut microbiota played a key role in KD-mediated epilepsy protection [378]. Another encouraging discovery was from Tantillo *et al.*, who reported that cytotoxic necrotizing factor (CNF1), a cytotoxin produced by *E. coli*, could be used to treat glioma by blocking cytokinesis in proliferating cells and causing senescence and death, thereby maintaining neural function [380]. Based on the above, some species of bacteria like gut microbiota may be capable of crossing the BBB to influence the behaviors of the brain. Although more investigations into the communication mechanism of these bacteria with the brain are still required, these discoveries undoubtedly open a new way to study the effects of bacteria on the brain. These research studies may inspire a bold idea to use bacteria for the treatment of other brain-related diseases, including Alzheimer's disease, Parkinson's disease, *etc*.

7. Current Challenges and Future Perspectives

For engineered microbial therapy, functional stability, clinical potency, and safety are essential factors that need to be considered for successful clinical translation. Using functional biomaterials to encapsulate engineered bacteria for oral delivery shows promise for targeted and controllable treatments of diseases, such as cancer, diabetes, and Crohn's disease. The selection of appropriate bacteria and biomaterial determines the safety and efficacy both *in vitro* and *in vivo*. However, several challenges, such as mucoadhesion and plasmid loss after oral administration, may affect the accuracy of bacterial localization, the release rate of therapeutics and the *in vivo* therapeutic efficacy, which must be extensively studied. Apart

from that, we also propose future perspectives for the oral delivery of bacteria in a broader range of applications.

7.1. Mucoadhesion

Recently, a novel concept for bacterial targeting, mucoadhesion, is proposed based on the close interactions between bacterial capsules and mucous layers [208]. The mucous layer, which provides a hydrophilic absorption barrier, is located on mucosal membranes that exist on the surfaces of epithelial tissues, including the respiratory, reproductive, and gastrointestinal tracts. This mucus layer is responsible for the exchange of gases, water, nutrients, and drugs with the underlying epithelium and the prevention of pathogen or toxic substance invasion [208]. Therefore, the delivery of therapeutics to pass through the mucus layer and attach to the mucosal membranes can prolong the drug residence time for enhanced efficiency of disease treatment. However, the therapeutic effect of the mucoadhesion delivery system is determined by its penetration efficiency, which is influenced by several factors, including mucus thickness, particle size, the dense fiber mesh in the mucus gel layer structure, and interactions between the particulates and the mucus [381].

Firstly, studies have shown that larger-sized particles could move more quickly in the mucus. However, Takeuchi *et al.*, demonstrated that both non-chitosan and chitosan-coated liposomes with a diameter of 100 nm could penetrate the mucus layer to a higher extent than those with larger sizes after oral administration [382]. This is because the dense fiber mesh (average mesh size of 100 nm) also significantly limits the movement of the larger particle [383]. Thus, size optimization of the delivery particles is key to obtaining elevated penetration efficiency. Secondly, the selection of materials for particle formation also affects their penetration efficiency in the mucus. Particles prepared by materials, such as alginate, pectin, and chitosan, have shown better bioavailability and longer residence time in the gastrointestinal tract [208]. Additionally, interactions between the particulates and the mucus

also determine their retention time in the target sites. The firm attachment of bacteria or bacteria-encapsulated microcapsules to mucosal membranes can guarantee long-term colonization for improved therapeutic efficiency [208]. Lectin molecules on the tip of bacterial type I pili are able to bind with mannose molecules on epithelial cells (in urinary and intestinal tracts) by the lectin-mannose bond, leading to the improved local concentration of drugs in the desired location and reduced side effects [383, 384]. This lectin-mannose bond provides one method to anchor bacteria for more precise targeting moieties. Collectively, taking the size and material properties of the particles into account allows the optimization of mucoadhesive capacity for gastrointestinal retention after oral administration.

7.2. Effective Encapsulation and Release

Encapsulation technology provides protection to minimize microbial death and maximize the therapeutic effect of microbes for oral delivery of bacteria, achieving an improved therapeutic effect [23]. There are some issues about bacteria encapsulation and release that need to be carefully considered.

Firstly, appropriate materials are required for the oral delivery of bacteria. Suitable materials offer protection with more space and higher survival rates for microbes in gastrointestinal environments. A combination of two or more types of biopolymers is the current trend for the selection of encapsulating materials, which can integrate the advantages of each polymer to achieve more effective protection and controlled release of bacteria for oral delivery. Furthermore, the materials must preserve bacterial fitness and motility [16]. The second challenge is to ensure the constant release of therapeutics at the disease site. To date, the choice of therapeutic bacteria to target disease areas is restricted to a relatively small range, such as gram-negative facultative anaerobes, which preferentially colonize anoxic regions of the tumor or some bacteria with auxiliary attractions like heat and magnetic force [162]. Additionally, bacterial flagella and pili provide energy for their active movement in liquid

media, which can sense environmental changes to achieve targeted delivery [16]. However, this targeted delivery is random and does not guarantee sufficient accumulation of bacteria at disease sites. Therefore, more strategies for targeting delivery of bacteria are required to extend the scope of their applications. Moreover, the sustained release of therapeutics in disease sites is also crucial for bacterial therapies. Synthetic biology and genetic engineering control bacterial behaviors through genetic circuits [30]. The periodic growth and lysis of bacterial populations enable the continuous release of biomolecules or drugs for long-term administration. However, genetic mutations that arise from this technology is still a question that needs to be considered and studied. As a therapeutic agent, bacteria are inevitably attacked by the host's immune system after administration, leading to severe immunogenicity. For example, *Salmonella*-based vehicles can be utilized for drug delivery systems to inhibit tumor growth [385]. However, the production of *Salmonella*-specific antibodies after administration reduces the tumor-targeting activity. Therefore, it is required to package the bacteria in biomaterials with excellent biocompatibility to minimize the interference of these antibodies, achieving the maximum therapeutic effect of bacterial therapy.

Overall, for effective bacterial therapy, the choice of encapsulation materials and technologies, as well as bacterial targeting and releasing abilities play a significant role in their applications in medicine.

7.3. Plasmid Loss

Genetic engineering endows bacterial cells with exogenous functions to improve the efficiency of bacteria therapies. However, the potential of mutation or plasmid loss may cause the development of resistance and microbiome dysbiosis, limiting the application of genetic bacteria circuits for disease treatment *in vivo* [17]. Genomic integration is one approach to prevent plasmid loss. Thus, a relatively stable method and several systems, including lambda red and CRISPR, have been developed [17]. Moreover, some stabilizing elements have been

inserted into engineered plasmids to prevent plasmid loss [30, 386]. Danino *et al.* engineered *E. coli* Nissle 1917 strain with a dual-stabilized maintenance plasmid system, containing a toxin-antitoxin system and the alp7 gene (**Fig. 17**) [60]. The toxin-antitoxin system could produce a toxin (hok) and a short-lived antitoxin (sok), so that cells could be killed by the toxin despite plasmid loss [387]. Alp7 was able to force plasmids to the poles of the cell to guarantee equal segregation during cell division [388]. Compared with the bacteria only modified with hok, dual-modified bacteria showed a smaller amount of plasmid loss after 24 hours or longer. In conclusion, the strategies that improve the stability of the plasmid need to be taken into consideration as much as possible.

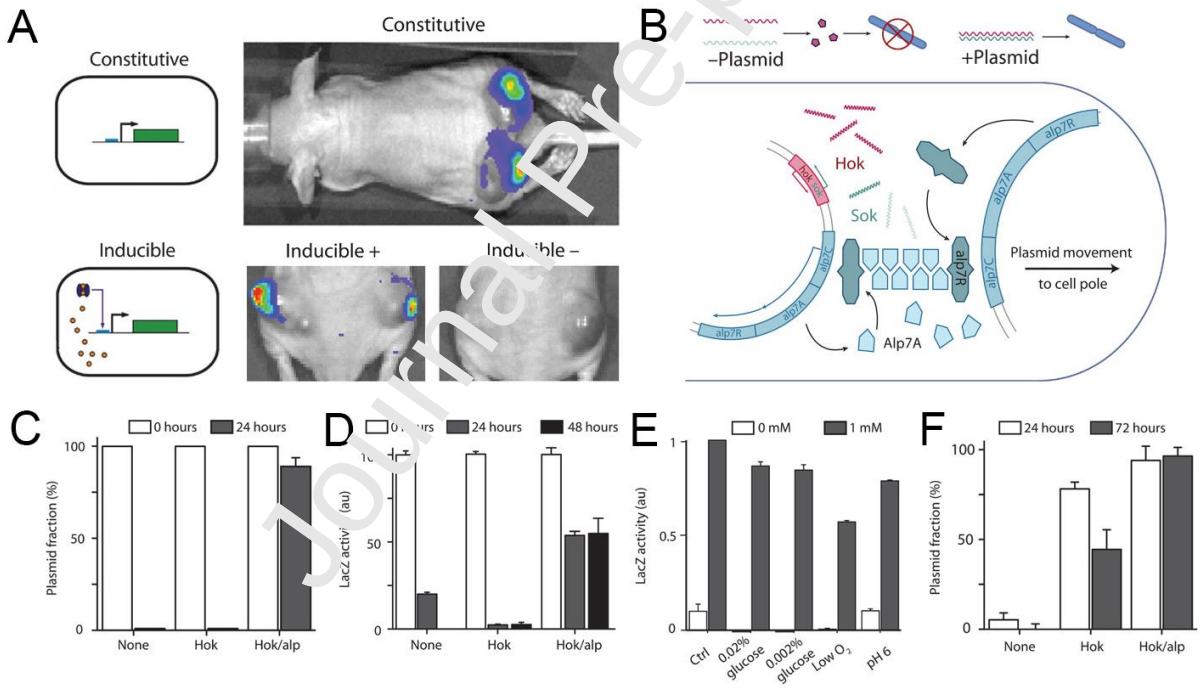

Fig. 17. The dually-stabilized vector effectively maintains PROP-Z activity *in vivo*. (A) Luciferase signals of bacteria equipped with a constitutive *luxCDABE* circuit or an AHL-induced *luxCDABE* circuit 24 h after intravenous injection in mice. (B) The dually-stabilized vector effectively maintains plasmid stability in the tumor. (C, D) The endurance of the lacZ plasmid evaluated by performing differential colony counts (C) and by examining lacZ enzymatic activity (D). (E) The activity of LacZ under different conditions. (F) The plasmid

fraction assessed after intravenous administration of bacteria in a subcutaneous tumor model. Reproduced with permission [60]. Copyright 2016, Science Translational Medicine.

7.4. Synergistic Strategy and Clinical Translation

The combination of bacterial therapy with other approaches is a rising trend in disease treatment. To date, applications of gut microbes have already achieved some clinical success in the regulation of immunotherapy, radiotherapy, chemotherapy [3, 10, 389-391]. However, there are still a notable proportion of cancer patients that do not respond to therapy [13]. Many mechanisms of immune evasion in tumors also remain to be learned. Furthermore, considerable efforts still need to be made to understand the mechanisms of immune cell dysfunction and tumor-associated local and systemic immune suppression.

Although numerous animal experiments have demonstrated the potential of bacterial treatment for many diseases like cancer and diabetes, some challenges are always required to be addressed before clinical evaluation. Firstly, an appropriate number of bacteria may be essential to produce enough drugs to induce therapeutic effects while also ensuring safety. Higher microbe concentrations potentially induce systemic toxicity [30]. Secondly, different targeting abilities of bacteria in patient groups cause differential efficacies that may affect the determination of effective treatment sites. Next, since genetic circuits may cause genetic mutations in the host, this potentially deleterious effect remains to be examined and controlled in animal models before any clinical trials are conducted on humans [8]. More importantly, it is essential to determine the correct combination of bacteria with other cancer treatments to achieve more effective tumor killing.

To address these issues, animal experiments need to be performed frequently and repeatedly before clinical trials. Additional basic studies are required to elucidate the function and interaction of host-associated bacterial communities. The insights gained from basic

research can drive the advancement of bacterial therapies into clinical trials and potentially result in the development of more effective treatments [392].

8. Conclusion

Here, we have outlined the design rules for the oral delivery of bacteria. A range of bacteria used for disease treatment, various encapsulation materials as well as technologies for oral bacterial delivery and their recent biomedical applications, are reviewed. For bacterial therapy, accurate targeting abilities, prolonged therapeutic-releasing time, and reduced side effects advance the development of novel biomedical methods for disease treatment. In addition, genetic engineering and synthetic biology provide more possibilities for modulating bacterial behaviors *in vivo*. Depended on these internal genetic engineering and external encapsulating strategies, bacteria have been developed as the advanced therapeutics or diagnostics for the management of a series of diseases. In the future, bacterial products applied in oral delivery should ideally display multiple characteristics and more intelligent functions, including specific targeting ability, environmental responsiveness, expression of therapeutic molecules, synergistic effect, *etc*. With the further understanding of the associations between bacteria and the host and persistent exploration of new encapsulating materials and techniques, novel bacterial therapies will achieve more stable functionality, enhanced therapeutic efficiency, and minimized side effects when supplementing *via* the oral route.

Acknowledgements

Shuqi Li, Weiqian Jiang and Chunxiong Zheng contributed equally to this work. This work was supported by the National Key Research and Development Program of China (2019YFA0111300), the Guangdong Province Science and Technology Innovation Special Fund (International Scientific Cooperation, 2018A050506035), and the National Natural Science Foundation of China (51903256, 21907113).

Declaration of Competing Interest

The author(s) declare no conflict of interest

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