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Co-encapsulation of probiotics with functional components: design strategies, synergistic mechanisms, biomedical applications, and challenges for industrialization

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Chronic diseases such as depression, diabetes, inflammatory bowel disease, and colorectal cancer are closely associated with gut microbiota dysbiosis and impaired intestinal barrier function. Probiotic supplementation represents an effective therapeutic approach for modulating gut microecology and alleviating disease symptoms. However, their limited survival rates and colonization efficiency in the gastrointestinal tract compromise their functional efficacy. Co-encapsulation of probiotics with functional components is an effective approach to enhance stability and has gradually become a major focus in current delivery system research. This review summarizes the co-encapsulation strategies of probiotics with functional components, including metabolites, prebiotics, and polyphenols. It also examines the applications of advanced manufacturing technologies such as microfluidics, 3D printing, layer-by-layer encapsulation, and electrospinning/electrospraying in this field. Through functional evaluation methods, including *ex vivo* gastrointestinal models, *in vivo* imaging, and metabolic tracking, the advantages of co-encapsulation in improving probiotic survival rates, targeted release capabilities, and functional stability have been demonstrated. Furthermore, this review explores the application potential of co-encapsulation in chronic disease intervention and identifies the challenges that remain in industrial scale-up, safety standardization, and clinical translation. This review aims to provide a scientific foundation for the clinical translation and industrial application of probiotic co-encapsulation technologies.

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1. Introduction

Probiotics are defined as live microorganisms that, when administered in adequate amounts (typically $\geq 10^6$–10^7 CFU g^{-1}), provide health benefits to the host. They are widely applied in modulating gut microbiota, maintaining barrier integrity, alleviating oxidative stress and inflammatory responses, and enhancing immune homeostasis.[1] Strains such as *Lacticaseibacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, and *Bifidobacterium longum* have demonstrated significant potential in the prevention and intervention of chronic diseases, including depression,[2] diabetes,[3] obesity,[4] inflammatory bowel disease,[5] and colon cancer.[6] However, most probiotics are highly sensitive to environmental factors such as gastric acid, bile salts, oxygen, enzymes, and thermal processing, resulting in limited survival rates and colonization efficiency during gastrointestinal transit, which severely constrains the realization of their health benefits.[7]

Co-encapsulation of probiotics with functional components represents an effective strategy to enhance stability and efficacy. Functional components, including metabolites (*e.g.*, short-chain fatty acids (SCFAs), tryptophan metabolites, and secondary bile acids), prebiotics (*e.g.*, inulin, fructo-oligosaccharides, and galacto-oligosaccharides), and polyphenols (*e.g.*, gallic acid, quercetin, and dihydromyricetin) can have complementary interactions with probiotics. These components not only serve as growth substrates by providing nutritional support and alleviating oxidative stress but also enhance targeted release efficiency and delay degradation processes, achieving synergistic effects where "1 + 1 > 2". Such co-encapsulation systems demonstrate superior protective effects and functional activities compared to single probiotic encapsulation.

Traditional encapsulation techniques, including emulsion, spray drying, freeze-drying, extrusion, and coacervation, can improve probiotic stability to some extent, but exhibit significant limitations in multi-component co-encapsulation, precise carrier structure control, and targeted release regulation.[7] In recent years, the emergence of advanced manufacturing technologies such as microfluidics,[8] 3D printing,[9] layer-by-layer (LbL) encapsulation,[10] and electrospinning/electrospraying[11] has provided novel solutions to address these challenges. These technologies enable precise design of carrier structures at molecular and microscopic scales, constructing complex encapsulation systems with multi-level responsive mechanisms and sequential release characteristics by controlling parameters such as carrier geometry, pore distribution, and wall material composition.

However, existing research on probiotic-functional component co-encapsulation has primarily focused on formulation design and improving survival rate, with limited in-depth elucidation of synergistic mechanisms and insufficient systematic evaluation of the application potential of advanced manufacturing technologies. Furthermore, comprehensive analyses of the therapeutic effects of co-encapsulation systems in chronic disease treatment and the technical challenges associated with industrial scale-up are lacking. Therefore, this review aims to: (1) elucidate co-encapsulation strategies and interaction mechanisms between probiotics and different functional components; (2) summarize application progress of advanced manufacturing technologies in probiotic co-encapsulation; (3) explore functional evaluation methods including *ex vivo* gastrointestinal simulation, *in vivo* imaging and metabolic tracking; (4) analyze the therapeutic potential of co-encapsulation systems in chronic disease intervention; (5) examine key challenges in industrial scale-up, providing

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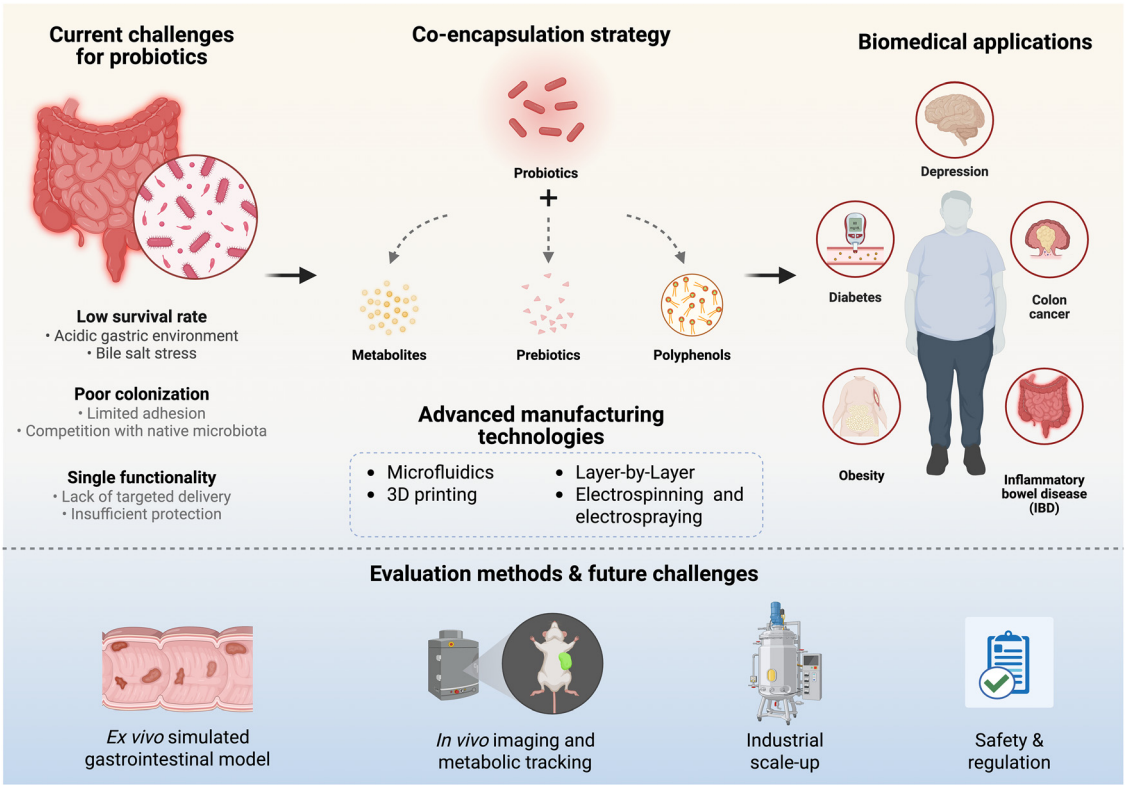

Fig. 1 Overview of co-encapsulation strategies for probiotics and functional components.

theoretical guidance for advancing the application of probiotic co-encapsulation technologies in biomedical fields (Fig. 1). This article is presented as a narrative review, primarily targeting researchers and specialists in the fields of biomaterials, nutritional sciences, and biomedical engineering. The literature search was conducted mainly using the Web of Science, Scopus, and PubMed databases. Keywords included "probiotics," "co-encapsulation," "functional components," "microfluidics," "3D printing," "layer-by-layer," and "electrospinning/electrospraying," in combination with terms such as "metabolites," "prebiotics," and "polyphenols." The search was limited to English-language peer-reviewed articles published between 2019 and 2025. The nomenclature of probiotic strains in this review follows the recent taxonomic revisions published in the List of Prokaryotic names with Standing in Nomenclature (LPSN) database. Exclusion criteria included conference abstracts, non-peer-reviewed sources, and studies not directly related to probiotic delivery systems.

2. Synergistic design of functional components

2.1. Co-encapsulation of metabolites and probiotics

Gut microbial metabolites are small molecules derived from the transformation of dietary components ingested by the host, endogenous substrates, or microbial metabolic processes, playing important signaling and regulatory roles in maintaining intestinal homeostasis and host health (Table 1). These metabolites not only participate extensively in energy metabolism, immune regulation, and mucosal barrier maintenance, but also profoundly influence the composition and dynamic succession of the intestinal microecology.[12] Currently well-studied representative metabolites include SCFAs, tryptophan metabolites, secondary bile acids, polyphenol derivatives, nitrogen-containing metabolites, vitamins, exopolysaccharides (EPS), and bacteriocins.[13] These metabolites interact with host cell surface or intracellular receptors (*e.g.*, G protein-coupled receptors, aryl hydrocarbon receptors (AhR), histone deacetylases) to trigger signaling cascades that regulate inflammation, barrier function, and metabolic pathways, while also providing feedback effects on the colonization capacity, metabolic activity, and stress adaptability of probiotics themselves.[14]

SCFAs, such as acetic acid, propionic acid, and butyric acid, are key metabolites produced by gut microbiota through the fermentation of dietary fiber. They play complementary roles in regulating the local environment, providing metabolic substrates, and maintaining microbial homeostasis.[15,33] Acetic acid is the most abundant of the SCFAs and can enter the bloodstream through diffusion, participating in lipid and cholesterol metabolism in the liver and peripheral tissues, and serving as an energy substrate to support the growth of probiotics such as *Lactobacillus* and *Bifidobacterium*. Propionic acid primarily regulates intestinal neural function and hunger hormone secretion by activating the GPR41 receptor, and inhibits the expansion of pathogenic bacteria in the colon, thereby promoting the ecological stability of probiotics. Butyric acid

Table 1 Regulatory mechanisms and signaling pathways of different types of metabolites on probiotics

Metabolites category	Representative molecules	Mechanisms of action	Representative probiotics	Regulatory effects on probiotics	Signaling pathways or targets	Ref.
Short-chain fatty acids (SCFAs)	Acetic acid, propionic acid, butyric acid	pH reduction, energy supply, immune regulation	*Lactobacillus acidophilus*, *Lacticaseibacillus casei*, *Bifidobacterium longum*	Promote growth, enhance colonization capacity	GPR41/43, HDAC inhibition	15–17
Tryptophan metabolites	Indole, indole-3-lactic acid, indole-3-propionic acid	Activate aryl hydrocarbon receptor (AhR) signaling pathway, upregulate tight junction protein expression, improve intestinal barrier function	*Lactiplantibacillus plantarum*, *Limosilactobacillus reuteri*, *Lactobacillus delbrueckii* subsp. *lactis*	Improve survival rate, enhance barrier response	AhR pathway	17–20
Secondary bile acids	Lithocholic acid, deoxycholic acid	FXR/TGR5-mediated immune and metabolic regulation	*Lactiplantibacillus plantarum*, *Bifidobacterium longum*, *Bacteroides thetaiotaomicron*	Enhance intestinal adaptability and anti-inflammatory properties	FXR, TGR5	21–23
Polyphenol metabolites	Protocatechuic acid, gallic acid	Degraded by intestinal microorganisms to generate small molecule metabolites, improve local redox status, assist energy supply	*Lacticaseibacillus casei*, *Bifidobacterium breve*	Promote bacterial growth, improve microbial structure	Nrf2, NF-κB	14, 24
Nitrogen-containing metabolites	γ-Aminobutyric acid (GABA), histamine	Activate GABA receptors or histamine receptors (H1R/H2R), participate in neuro-immune regulation	*Lactobacillus helveticus*, *Bifidobacterium bifidum*	Enhance stress tolerance and regulate functional status	GABA receptor, H1R/H2R	25
Vitamins	B vitamins, K vitamins	Microbial regulation, synthesis assistance, immune balance	*Bifidobacterium adolescentis*	Enhance ecological stability and microbial interactions	One-carbon metabolism, folate cycle, methylation pathway	12, 26
Exopolysaccharides (EPS)	Dextran, rhamnose-mannose polysaccharide, mannan, glucomannan	Form protective biofilms on intestinal surface, improve acid and bile salt tolerance	*Lacticaseibacillus rhamnosus* GG	Enhance adhesion capacity, improve stress adaptability	TLR2, NOD2	27–29
Bacteriocins	Nisin, Plantacin B	Enhance probiotic competitive advantage by inhibiting pathogen proliferation	*Lactococcus lactis*, *Lactiplantibacillus plantarum*, *Enterococcus faecium*	Improve colonization capacity and microecological adaptability	Membrane permeability disruption	30–32

is the major energy source for colonic epithelial cells, enhancing the colonization capacity of probiotics, and regulates intestinal barrier function and host immune status by inhibiting histone deacetylase (HDAC).[15–17] Tryptophan metabolites such as indole, indole-3-lactic acid, and indole-3-propionic acid activate the AhR signaling pathway to upregulate tight junction protein expression, improve barrier function, and reduce inflammation levels, thereby creating a favorable environment for the colonization and survival of probiotics.[17,18] These effects have been verified in strains including *Lactiplantibacillus plantarum*, *Limosilactobacillus reuteri*, and *Lactobacillus delbrueckii* subsp. *lactis*.[19,20] Secondary bile acids (*e.g.*, lithocholic acid and deoxycholic acid) are converted from primary bile acids under microbial action and can bind to FXR and TGR5 receptors to regulate inflammatory responses and bile acid circulation, enhancing the stress adaptability of strains such as *Lactiplantibacillus plantarum* to the intestinal mucosa.[21,22] Although polyphenol metabolites are difficult to absorb directly, they can be degraded by microorganisms in the colon into small-molecule metabolites, such as protocatechuic acid and gallic acid. These substances can be further utilized as energy sources by bacteria such as *Lacticaseibacillus casei* and *Bifidobacterium breve*, and can also improve microbial structure by regulating local redox status. However, the underlying mechanisms of these effects require further investigation.[14,24] Nitrogen-containing metabolites such as γ-aminobutyric acid (GABA) and histamine participate in neuro-immune regulation by activating GABA receptors or histamine H1R/H2R receptors, showing potential for enhancing stress tolerance and regulating functional status in bacteria such as *Lactobacillus helveticus* and *Bifidobacterium bifidum*.[25] In addition, gut symbiotic bacteria can synthesize various vitamins, such as B vitamins and K vitamins. For example, *Bifidobacterium adolescentis* can synthesize vitamin B12, which enhances the ecological stability of probiotics by maintaining microbial diversity and regulating host immune responses.[12] EPS are high molecular weight metabolites secreted by certain probiotics that can form protective adhesive films in the intestine, improving acid and bile salt tolerance and promoting adhesion. EPS secreted by *Lacticaseibacillus rhamnosus* GG helps it form stable biofilms in the colon, facilitating long-term symbiosis.[27,28] Bacteriocins are antimicrobial small peptides synthesized by probiotics. For instance, nisin can inhibit pathogen proliferation, providing competitive advantages for bacteria such as *Lactococcus lactis* and synergistically enhancing their colonization capacity in microecological environments.[30,31]

Based on the complementary mechanisms of metabolites in metabolic regulation and intestinal microecological intervention, researchers have co-encapsulated them with probiotics to enhance the stability and functional capacity of composite systems in the gastrointestinal environment. Pandey *et al.* employed a two-step ultrasonication technique using dextran and whey protein as encapsulating materials to prepare GABA and lactic acid bacteria co-encapsulated double emulsion (W1/O/W2) microcapsules, where GABA and lactic acid bacteria were co-distributed in the inner aqueous phase (W1), followed by ultrasonic emulsification to form water-in-oil emulsion and subsequent emulsification into an outer aqueous phase containing dextran or whey protein to form the double emulsion structure (Fig. 2A). The surface-active properties of GABA reduced the primary emulsion droplet size to 1.5–3 μm, and the co-encapsulated lactic acid bacteria maintained viability of 10^5–10^7 CFU mL^{-1} under simulated gastrointestinal conditions, while free bacterial strains were completely inactivated within 2 hours in gastric fluid.[34] This research team further utilized spray-drying technology to co-encapsulate GABA and *Lactiplantibacillus plantarum* in an outer polysaccharide matrix composed of inulin, dextran, and maltodextrin (Fig. 2B). The optimized wall material combination (0.4% inulin, 4.6% dextran, 8.4% maltodextrin) achieved 84.22% GABA encapsulation efficiency and 99.21% lactic acid bacteria encapsulation efficiency.[35] Srivastava *et al.* co-encapsulated vitamin B9 with *Bacillus coagulans* spores in a chitosan/gellan gum/κ-carrageenan tri-composite hydrogel, achieving segmented release in the gastrointestinal environment.[36] In simulated gastric fluid (SGF), the release rates of vitamin B9 and spores were 48.3% and 2.8%, respectively, which increased to 52.5% and 11.2% in simulated intestinal fluid (SIF), with spore-to-vegetative cell conversion and colonization observed. Compared to the free-form group, co-encapsulation was more favorable for maintaining spore viability and vitamin B9 stability throughout the entire digestion process. These studies demonstrate that metabolites not only regulate host responses in the intestine but can also serve as functional auxiliary factors, improving the encapsulation efficiency, stability, and controlled release performance of probiotic delivery systems.

2.2. Co-encapsulation of prebiotics and probiotics

Prebiotics are defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as ''substrates that are selectively utilized by host microorganisms conferring a health benefit''. Qualified prebiotics must satisfy the following criteria: resistance to host enzymatic digestion and gastric acid hydrolysis in the upper digestive tract, selective fermentation by colonic microbiota, and stimulation of the growth and activity of beneficial intestinal bacteria to exert health effects.[37] According to differences in chemical structure and origin, prebiotics can be classified into four categories:[38,39] (1) inulin-type fructans, including inulin (degree of polymerization 2–60) and fructo-oligosaccharides (degree of polymerization 2–10), are composed of fructose units linked by β-(2→1) glycosidic bonds and are primarily derived from plants such as chicory and Jerusalem artichoke. (2) Oligosaccharides, including galacto-oligosaccharides (β-1,4 and β-1,6), xylo-oligosaccharides (β-1,4), and isomalto-oligosaccharides (predominantly α-1,6), which are mainly obtained through enzymatic hydrolysis of starch or plant polysaccharides. Human milk oligosaccharides are complex oligosaccharides derived from human milk, containing fucose and sialic acid modifications, with potential prebiotic functions. (3) Resistant starches (RS), including RS2 (high-amylose starch), RS3 (retrograded starch formed by cooling), and RS4 (chemically modified starch *via* esterification or crosslinking), can resist enzymatic digestion by small intestinal α-amylase and are fermentable in

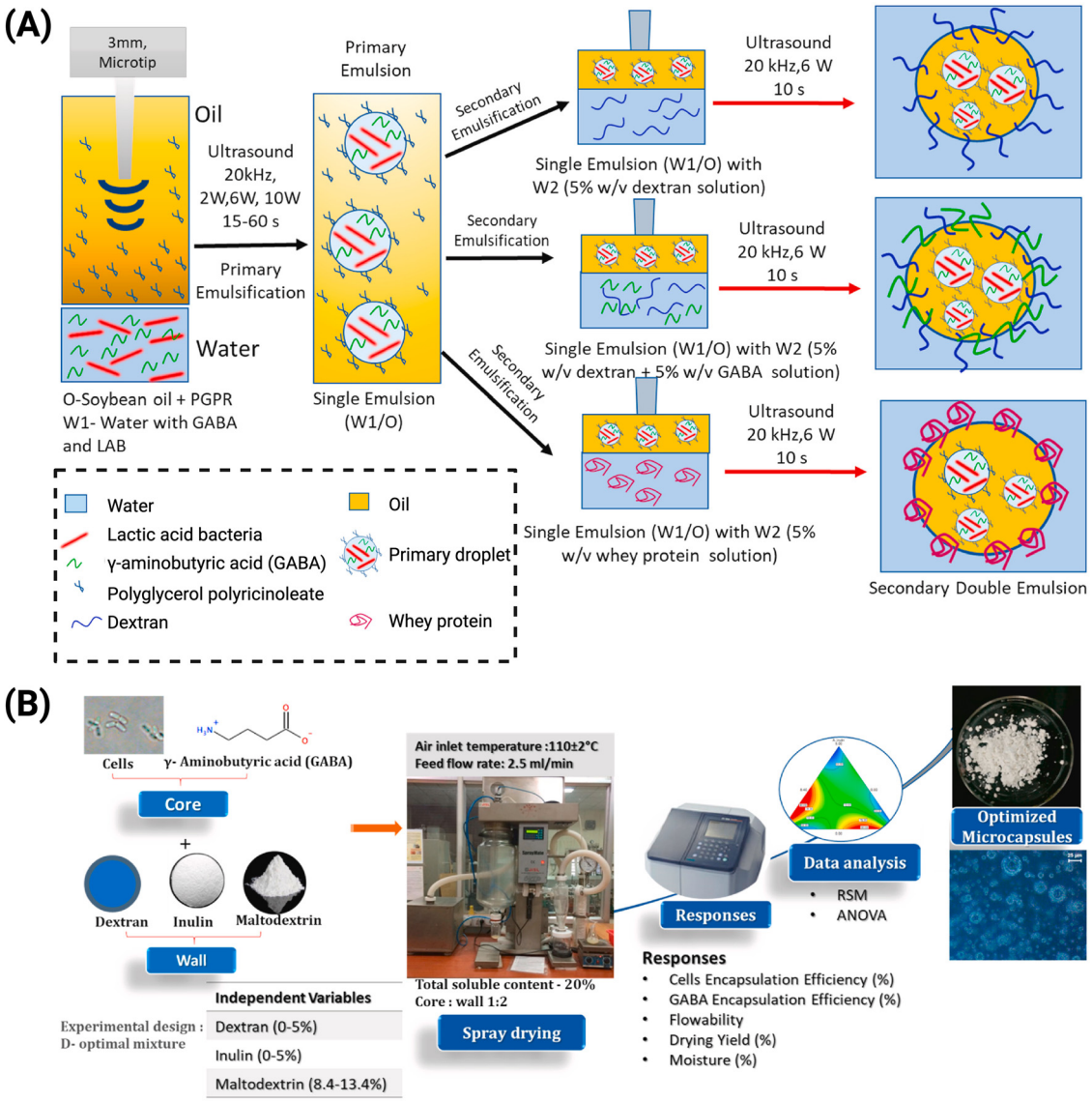

Fig. 2 Co-encapsulation strategies of probiotics with metabolites. (A) Process flow of two-step ultrasonication for preparing GABA and lactic acid bacteria co-encapsulated double emulsion microcapsules, reproduced from ref. 34 with permission from Elsevier, copyright 2021. (B) Spray-drying microencapsulation of GABA and *Lactiplantibacillus plantarum*, reproduced from ref. 35 with permission from Elsevier, copyright 2021.

the colon. (4) Other functional fibers, such as β-glucan, pectin, and arabinoxylan, are non-starch polysaccharides with distinct backbone linkages and side-chain modifications, and can be utilized by specific microbial groups to produce SCFAs.[40–43]

Prebiotics promote the growth, metabolism, and functionality of probiotics through multiple pathways.[44] First, prebiotics, as indigestible carbohydrates, can resist gastric acid and are not degraded or absorbed by mammalian enzymes, reaching the large intestine intact where they are selectively fermented by gut microbiota. During fermentation, probiotics such as *Bifidobacterium* and *Lactobacillus* can specifically recognize and utilize these prebiotic molecules, converting them into required carbon sources through specialized enzyme systems.[45] Second, fermentation process produces SCFAs (mainly comprising acetic, propionic, and butyric acids). These metabolites not only provide energy for intestinal epithelial cells but also lower intestinal pH, creating a microenvironment favorable for probiotic growth while inhibiting pathogenic bacteria.[46] Meanwhile, prebiotics help regulate oxygen concentration in the intestinal environment by consuming oxygen, providing more suitable survival conditions for anaerobic probiotics. Additionally, prebiotics can bind with bile acids and promote their degradation, reducing bile acid damage to probiotic cell membranes and thereby improving probiotic survival during gastrointestinal transit. Finally, the degree of polymerization of prebiotics directly affects their utilization efficiency by probiotics. Generally, prebiotics with lower degrees of polymerization are more easily and rapidly utilized, preferentially stimulating probiotic proliferation.[47] It has been reported that short-chain inulin (degree of polymerization 4–5) can be rapidly utilized by

Bifidobacterium and promote its growth, whereas long-chain inulin (degree of polymerization 23–25) requires initial decomposition by strains with complex polysaccharide-degrading capabilities, such as *Bacteroides*, before being utilized by other probiotics.[48]

To date, numerous studies have investigated the co-encapsulation of prebiotics and probiotics within microcapsules. Zaeim *et al.* co-encapsulated *Bifidobacterium lactis* and *Lactiplantibacillus plantarum* with resistant starch or inulin, respectively, in calcium alginate-chitosan microcapsules. Resistant starch enhanced the survival rate of *Bifidobacterium lactis* to 7.19 ± 0.15 log CFU g^{-1} in simulated gastrointestinal environments, while long-chain inulin maintained *Lactiplantibacillus plantarum* viability at 6.33 ± 0.21 log CFU g^{-1} after 90 days of storage at 25 °C.[49] In another study, Liao *et al.* co-encapsulated *Lactobacillus fermentum* with different types of oligosaccharides (galacto-oligosaccharides, isomalto-oligosaccharides, fructo-oligosaccharides, and xylo-oligosaccharides) in calcium alginate microcapsules. The encapsulation efficiency of all co-encapsulation groups was higher than that of the calcium alginate alone group (79.52–89.75% *vs.* 78.37%), and the microcapsules remained stable in simulated gastric fluid while releasing probiotics in the intestinal environment. All types of oligosaccharides improved the gastrointestinal survival of *Lactobacillus fermentum* to varying degrees, with the fructo-oligosaccharide group showing optimal effects. After 4 hours of sequential gastric and intestinal fluid treatment, bacterial viability was 8.53 ± 0.23 log CFU g^{-1}, compared to 4.99 ± 0.19 and 3.45 ± 0.49 log CFU g^{-1} for the single-encapsulation and free-strain groups, respectively.[50] Furthermore, Raddatz *et al.* employed internal gelation combined with freeze-drying to construct probiotic-prebiotic co-encapsulated microcapsules containing *Lactobacillus acidophilus* LA-5, with prebiotics including hi-maize, inulin, and rice bran (Fig. 3A).[51] Under storage at 25 °C, the viable counts in the co-encapsulation systems were maintained for 90 days (inulin, 6.35 ± 0.02 log CFU g^{-1}), 75 days (hi-maize, 6.6 ± 0.1 log CFU g^{-1}), and 75 days (rice bran, 6.1 ± 0.0 log CFU g^{-1}), while the control group without prebiotics lost viability after 60 days. In further research, Ji *et al.* co-encapsulated β-glucan with *Lactiplantibacillus plantarum*, where β-glucan was fermented by intestinal microbiota in the colon to produce SCFAs, providing synergistic effects with the released probiotics to exert anti-inflammatory and gut microbiota-regulating functions (Fig. 3B).[52]

2.3. Co-encapsulation of polyphenols and probiotics

Polyphenols are secondary metabolites produced by plants, including phenolic acids, flavonoids, and tannins, which are beneficial to human health and have various important physiological activities such as antioxidation,[53] anti-tumor,[54] and anti-inflammation.[55] The complex structure of polyphenols makes them difficult to hydrolyze in the gastrointestinal tract and unable to enter the bloodstream to reach the corresponding targets, thus offering certain advantages in stability and modification.[56] Polyphenols do not always exhibit high biological activity after consumption and have limited absorption and utilization in the human body. However, the rich microbial enzyme library in the colon can effectively degrade dietary polyphenols into small molecules with higher physiological activity, and the secondary metabolites produced from the decomposition of polyphenols by probiotics promote the growth of probiotics.[57] For example, chocolate polyphenols can be utilized by probiotics as substrates for metabolism. Hossain *et al.* used freeze-drying and embedding technology to add *Lactobacillus delbrueckii* subsp. *bulgaricus* to chocolate with 45% and 70% cocoa content.[58] They found that compared with 45% cocoa chocolate, encapsulated probiotics in 70% cocoa chocolate produced significantly more acetic acid, propionic acid, isobutyric acid, butyric acid, and isovaleric acid. This suggests that chocolate with higher cocoa content is more conducive to the production of SCFAs, and probiotic chocolate may serve as an excellent nutritional source for the intestinal microbiota.

Numerous studies have indicated that the antioxidant activity of polyphenols is closely related to their structural characteristics (such as the number of hydroxyl groups, orthodiphenolic groups, 4-oxo structures, *etc.*), and polyphenols can act synergistically with probiotics to exert antioxidant effects by scavenging free radicals such as $ABTS^{+}$ and DPPH. Fruits are rich in conjugated polyphenols, which generally exhibit relatively low antioxidant and biological activities. The enzymes produced by probiotics during fermentation can catalyze deglycosylation, demethylation, and hydrolysis to convert these conjugated polyphenols into aglycone flavonoids and phenolic acids with higher bioactivity. This transformation enhances hydrogen-donating capacity and electron transfer, leading to increased antioxidant activity.[59] Ma *et al.* fabricated polyvinyl alcohol (PVA)/fucoidan (FUC) blend nanofibers *via* electrospinning to co-encapsulate the probiotic *Lactiplantibacillus plantarum* 69-2 (LP69-2) along with four polyphenols (gallic acid, chlorogenic acid, dihydromyricetin, and hesperidin).[60] The results demonstrated that the survival rate of probiotics (still over 7 lg CFU g^{-1} after 21 days) and the antioxidant activity of nanofibers (DPPH scavenging rate up to 53.49%) were significantly improved, and thermal stability of the system was enhanced, offering a novel strategy for the development of functional foods.

In addition, polysaccharides possessing prebiotic activity can be utilized to fabricate microcapsule systems and stabilize their structural strength. Pectin, a polysaccharide extracted from plant cell walls, can be employed for the microencapsulation of bioactive compounds such as polyphenols.[61] Sun *et al.* employed pectin, alginate (WGCA@LK), and Fujian brick tea polysaccharide (WGCF@LK) as wall materials and combined with chitosan-whey protein isolate through LbL coagulation reactions to construct co-microcapsules of polyphenols and probiotics (Fig. 4A).[62] The results revealed that WGCA@LK increased the encapsulation rate of polyphenols (42.41%) and improved the survival rate of probiotics in the acidic gastric environment and during storage. The prebiotic activity of WGCF@LK supported probiotic growth, and the microcapsules themselves good antioxidant and anti-inflammatory effects. Furthermore, the type of polyphenol used can significantly affect the properties and efficacy of the encapsulation system. Zhu *et al.* found that encapsulating *Lactiplantibacillus plantarum*

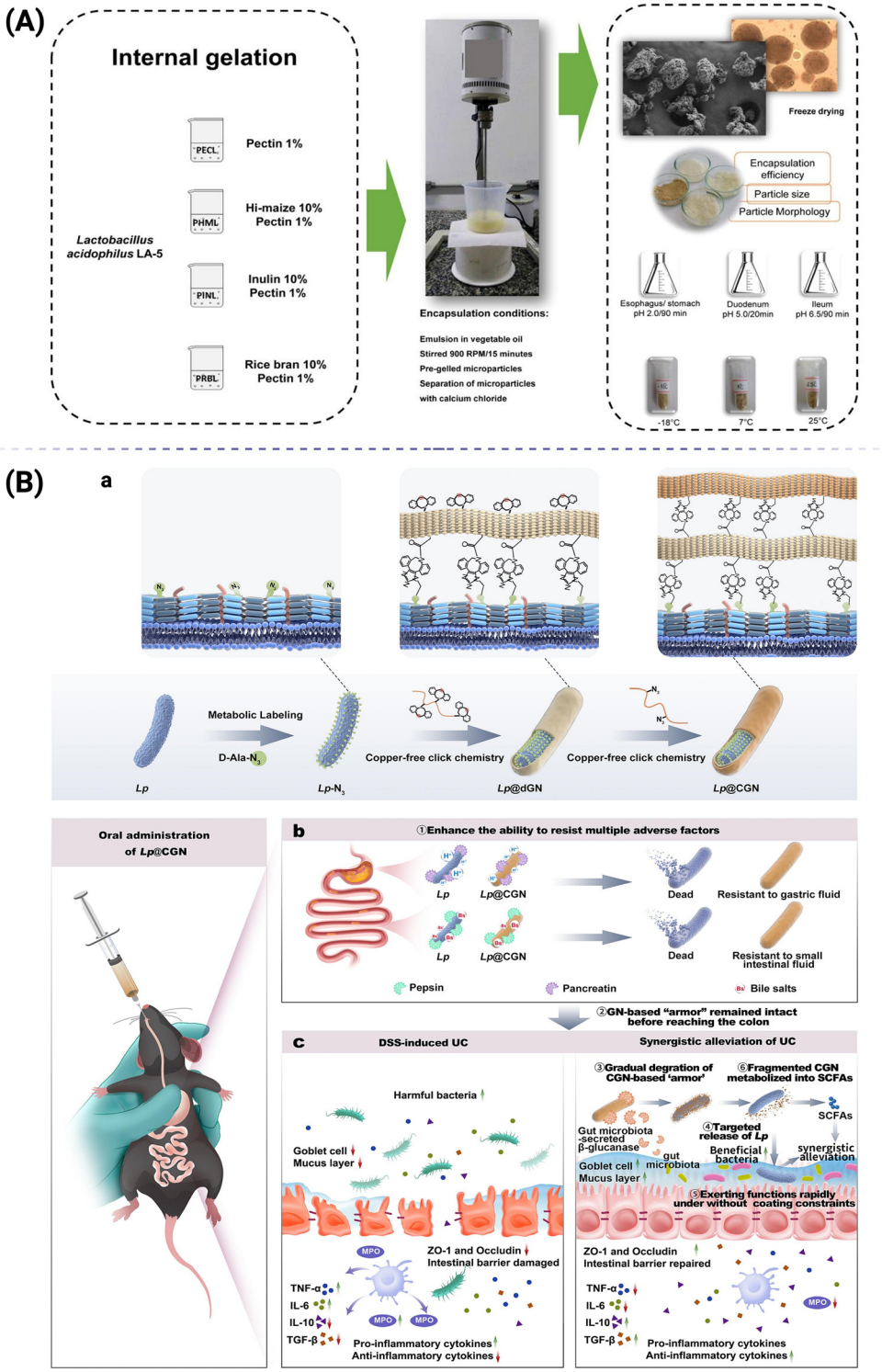

Fig. 3 Co-encapsulation of probiotics with prebiotics. (A) Microcapsules of *Lactobacillus acidophilus* LA-5 co-encapsulated with pectin and different prebiotics: hi-maize (PHML), inulin (PINL), and rice bran (PRBL), reproduced from ref. 51 with permission from Elsevier, copyright 2020. (B) Schematic of *Lactiplantibacillus plantarum* co-encapsulated with β-glucan *via* bioorthogonal coating and its colon-targeted delivery mechanism, reproduced from ref. 52 with permission from Elsevier, copyright 2025.

in a novel C-phycocyanin-pectin-based hydrogel could enhance the bacterium's survival under gastrointestinal conditions (from 5.7 log CFU to 7.1 log CFU).[63] Increasing of C-phycocyanin concentration enhanced the hydrogel's mechanical strength and stability. Furthermore, incorporating resveratrol (RES) or tannic acid (TA) increased the hydrogel's hardness (to 608.3 g

and 637.0 g, respectively) and its water-holding capacity (to 94.2% and 94.8%, respectively), compared to the control (595.4 g and 93.9%, respectively).

Co-delivery systems for polyphenols and probiotics can protect against the adverse effects of antibiotics. Zhang *et al.* prepared a pH-responsive microgel for the co-delivery of *Weizmannia coagulans* BC99 and proanthocyanidins (PCs) (Fig. 4B).[64] Interactions among PCs, pectin, and protein within the microgel maintained the viability and tolerance of BC99 in complex microenvironments (such as H_2O_2 and antibiotics). Furthermore, the microgel achieved targeted release of the probiotics in the neutral intestinal fluid. Pan *et al.* utilized the adhesion property of plant polyphenols at the cell interface to design a probiotic nanoshield capable of maintaining high biological activity during antibiotic exposure.[65] In this case, the polyphenol nanoshield adsorbed antibiotics around probiotics through multiple interactions between plant polyphenols and antibiotics, thereby protecting probiotics, significantly reducing antibiotic-associated diarrhea and improving intestinal flora balance.

The co-encapsulation strategy of probiotics and polyphenols can overcome their sensitivity to pH, temperature and other conditions. This approach reduces processing costs, improve encapsulation efficiency, reduce polyphenol degradation and survival loss of probiotics, achieve targeted release in the colon, and exert synergistic effects *in vivo*.[66] On the one hand, polyphenols can act as prebiotics to promote the proliferation of probiotics. Zhang *et al.* employed 16S rRNA sequencing to reveal that betel nut polyphenols increased the abundance of multiple probiotics and effectively inhibited harmful bacteria.[67] Ma *et al.* used a complex of zein and chitosan (ZCSC) to co-deliver *Lacticaseibacillus casei* and polyphenols. They found that a low concentration of quercetin (0.05%) could increase probiotic activity (over 6.23×10^9 CFU mL^{-1}) and encapsulation efficiency (>19.3%), further promoting the growth of probiotics.[68] On the other hand, polyphenols can enhance the colonization ability of the microbiota in the intestine. Fang *et al.* first utilized the adsorption *via* hydrogen bonding between a metal-tea polyphenol network and modified gelatin (GelAGE) to form a dense protective coating on the probiotic surface through a thiol-ene photo-click reaction. This simplified and efficient coating can significantly enhance probiotic viability in gastrointestinal fluid and increase their intestinal colonization rate and persistence.[69]

3. Application of advanced manufacturing technologies in probiotic co-encapsulation

As the role of probiotics in disease intervention and intestinal microecological regulation has gained increasing attention, higher requirements are being placed on the structural design and functional performance of their delivery systems. Traditional encapsulation methods, such as spray drying, freeze-drying, and ionic gelation, have contributed to improving

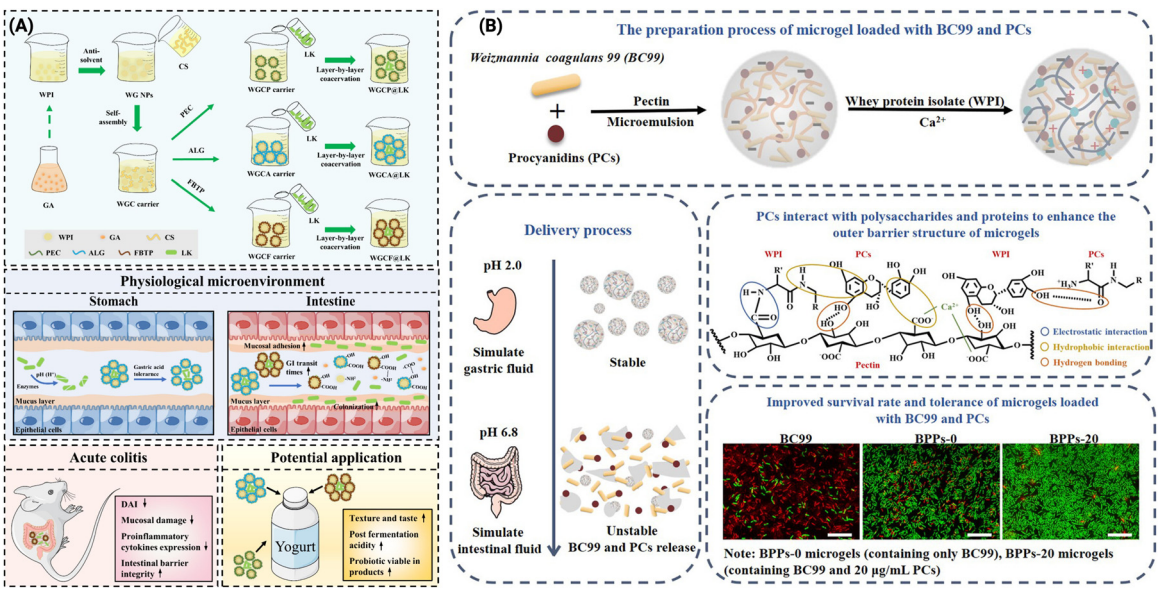

Fig. 4 Co-encapsulation of probiotics with polyphenols. (A) Schematic illustration of co-encapsulation of polyphenols and probiotics using chitosan-whey protein isolate-gallic acid (WGC) nanoparticles as the inner carrier. Through layer-by-layer coacervation with pectin (WGCP@LK), alginate (WGCA@LK), and Fu brick tea polysaccharide (WGCF@LK), different co-microcapsules were constructed to encapsulate *Lactobacillus kefiranofaciens* (LK) and polyphenols. The middle panel illustrates the resistance of microcapsules under gastric and intestinal conditions and their colonization in the gut. The bottom panel shows their efficacy in alleviating acute colitis in mice and potential application in probiotic yogurt. WPI, whey protein isolate; GA, gallic acid; CS, chitosan; PEC, pectin; ALG, alginate; FBTP, Fu brick tea polysaccharide; LK, *Lactobacillus kefiranofaciens*, reproduced from ref. 62 with permission from Elsevier, copyright 2024. (B) A pH-responsive microgel composed of pectin, whey protein, and Ca^{2+} enabled the co-encapsulation of *Weizmannia coagulans* BC99 and procyanidins to enhance probiotic tolerance and survival, reproduced from ref. 64 with permission from Elsevier, copyright 2025.

probiotic stability, but still exhibit significant limitations in size control, functional integration, and multi-component co-delivery.[8,44,46] To achieve more precise sustained release, targeted delivery, and controlled design of carrier structures, various advanced manufacturing technologies have been introduced into the construction of probiotic delivery systems in recent years.

This section introduces four representative advanced encapsulation technologies, including microfluidics, 3D printing, LbL encapsulation, and electrospinning/electrospraying methods. These technologies demonstrate different advantages in microstructural regulation, composite material fabrication, and encapsulation efficiency enhancement (Table 2). Through analysis of their principal mechanisms, technical parameters, application cases, and potential challenges, technical references can be provided for the co-encapsulation of probiotics with functional components.

3.1. Microfluidics

Microfluidic technology enables precise control of droplet size and composition by regulating fluid dynamic behavior in micrometer-scale channels.[77] Based on device geometry, microfluidic devices for droplet generation mainly include T-type microreactors (channel width 20–200 μm), flow-focusing microreactors (focusing orifice 10–100 μm), and co-flowing microreactors (inner-to-outer tube diameter ratio of 1 : 2–1 : 10), which can produce monodisperse droplets in the range of 1–1000 μm (Fig. 5(A)).[70,109,110] The core principle of these devices is based on laminar flow control and interfacial phenomena. At the microscale, Reynolds numbers are relatively low (typically $Re < 100$), and fluids exhibit stable laminar flow behavior, where mixing between different phases relies primarily on molecular diffusion rather than convective mixing. The mechanism of microfluidic droplet generation is mainly achieved by controlling the flow rate ratio, viscosity, and channel geometric parameters between the continuous and dispersed phases.[111] When the dispersed phase enters the continuous phase region, necking and droplet break-up occur under the combined effects of interfacial tension, shear stress, and pressure gradient, forming monodisperse droplets.[112] Droplet size typically follows classical dimensionless number relationships such as the Capillary number or the Weber number, allowing precise control from the nanometer to the submillimeter scale through adjustment of fluid properties and operating parameters.[113,114]

In probiotic encapsulation, antagonistic interactions between different strains and environmental stresses often lead to a loss of viability.[115] Microfluidic technology can precisely control the microenvironment within individual droplets, allowing different probiotic strains to be individually encapsulated in independent microcapsules to form dual-core or multi-core structures, achieving physical separation to avoid inter-strain interference.[116–118] Moreover, microspheres produced by microfluidics exhibit high size uniformity (typically with a polydispersity index below 0.1), and such monodispersity facilitates the prediction of probiotic release kinetics in the gastrointestinal tract.[119] Zhao *et al.* employed an electrostatically driven microfluidic technology to prepare dual-core microcapsules, encapsulating *Lactobacillus* and *Bacillus subtilis* separately into isolated compartments to avoid antagonistic effects that occurred after the third day during direct co-culture, as shown in Fig. 5(B(a)).[71] This system limited the diffusion of acidic metabolites through physical separation, resulting in approximately a 20% increase in probiotic proliferation compared to the direct mixing group. In a mouse model of metabolic syndrome, the dual-core microcapsule group showed better outcomes than the free probiotic group in terms of reducing fat deposition in the liver, restoring intestinal barrier function, and alleviating inflammation.

The effectiveness of probiotic encapsulation *via* microfluidics relies on the coordinated control of multiple process parameters. An increased flow rate ratio between the continuous and dispersed phases enhances shear stress, resulting in a power-law or near-logarithmic reduction in droplet size.[72] Wang *et al.* used a gas–liquid microfluidic chip to fabricate kelp nanocellulose/sodium alginate microcapsules encapsulating *Lactobacillus paracasei* LC-01.[73] By adjusting the needle height (2–14 cm), flow rate (2.4–7.2 mL h^{-1}), and $CaCl_2$ bath rotation speed (300–1100 rpm), the microcapsule diameter was precisely controlled within 250–550 μm, achieving an encapsulation efficiency of up to 96.11%. Similarly, Luo *et al.* employed a polymethyl methacrylate (PMMA) microfluidic chip to produce cysteine-modified chitosan microspheres loaded with *Bifidobacterium* FL-276.1 (Fig. 5(B(b))).[74] When the flow rate ratio between the dispersed phase (bacteria-containing chitosan solution) and the continuous phase (paraffin oil system) was maintained at 1 : 6, monodisperse microspheres with diameters of 230–395 μm were obtained. The encapsulation efficiency reached 80%, and the survival rate after SGF treatment was 92%, compared with only 21% for free cells, indicating a marked improvement in acid resistance. For complex delivery requirements, core–shell structures further enhance the functionality of the encapsulation system. Wang *et al.* employed multilayer core–shell microcapsules using microfluidics, where the inner core was a Pickering emulsion containing *Lactiplantibacillus plantarum* Lp90, the middle layer was a composite shell of sodium alginate and kelp nanocellulose, and the outer layer was acetic acid-containing corn oil.[75] By controlling the three-phase flow rates (500 μL h^{-1}, 800 μL h^{-1}, and 3600 μL h^{-1}), they obtained an encapsulation efficiency of 95.95%.

In summary, microfluidic technology enables the construction of highly uniform microcapsules and multilayer core–shell structures, showing great potential for improving probiotic survival and functional retention. However, current devices are limited to laboratory-scale applications, with high chip fabrication costs and insufficient standardization and automation of equipment.[76] Future research should focus on scale-up production, optimization of multi-strain co-encapsulation designs, and co-encapsulation strategies that integrate probiotics with functional components.

Table 2 Comparative analysis of advanced manufacturing technologies for probiotic encapsulation

Technology	Typical encapsulation efficiency	Viability retention	Scalability	Cost	Throughput	Material limitations	Key advantages	Key disadvantages	Ref.
Microfluidics	80–96.11%	92%	Low	High	Low	• Requires precise control of flow rate ratios between continuous and dispersed phases • Relies on oil-based systems or specific polymers (*e.g.*, PMMA)	• Precise size control (1–1000 μm) • High monodispersity (PDI <0.1) • Capability to create dual-core/multi-core structures to avoid inter-strain antagonism	• Complex equipment with high manufacturing costs • Limited production capacity, difficult to industrialize • Equipment standardization and automation levels need improvement	70–77
3D Printing	N.R.	80.40–98%	Medium	Medium	Low-Medium	• Limited range of food-grade bio-inks (mostly polysaccharides/proteins; viscosity requirements narrow)	• Potential for personalized nutrition and functional food design • Capability for complex geometry fabrication	• Limited printing speed • Potential cell damage due to mechanical stress and heat during printing	78–85
Layer-by-Layer	78–92%	60–90.16%	Medium	Medium	Medium	• Requires oppositely charged macromolecular materials (polycations and polyanions) • Faces challenges in biocompatibility selection of encapsulation materials • Stability of multilayer films over long-term storage can be problematic	• Precise nanometer-scale control of film thickness and permeability • High protection against acid, bile salts, and oxidative stress • Versatility in combining multiple functional materials (proteins, polysaccharides, lipids)	• Multi-step layer-by-layer deposition process is complex and time-consuming • Requires optimization of multiple process parameters (ionic strength, pH, polymer concentration, deposition time)	7, 86–93
Electrospinning	N.R.	62.79–91.96%	Medium	Low-Medium	Medium	• Organic solvent residue • Polymer molecular weight and viscosity requirements	• High surface area • Fiber continuity • Core–shell structure enables pH-responsive release	• Solvent rapid evaporation and osmotic stress • High voltage requirement and potential impact on cells • Process parameter sensitivity (voltage, distance, flow rate, humidity, *etc.*)	8,44, 94–100
Electrospraying	98%	Dry: 46.7–56.34%; Wet: 86.46-97.25%	Medium	Low-Medium	Medium-High	• Wet method limited to ionic crosslinking materials • Food-grade materials require synthetic polymers or organic solvents due to insufficient spinnability	• Uniform spherical particles • Wet method avoids organic solvents • Simple operation	• Solvent residue issues • Complex process optimization • High sensitivity to salt stress	49, 101–108

Note: N.R.: Not Reported.

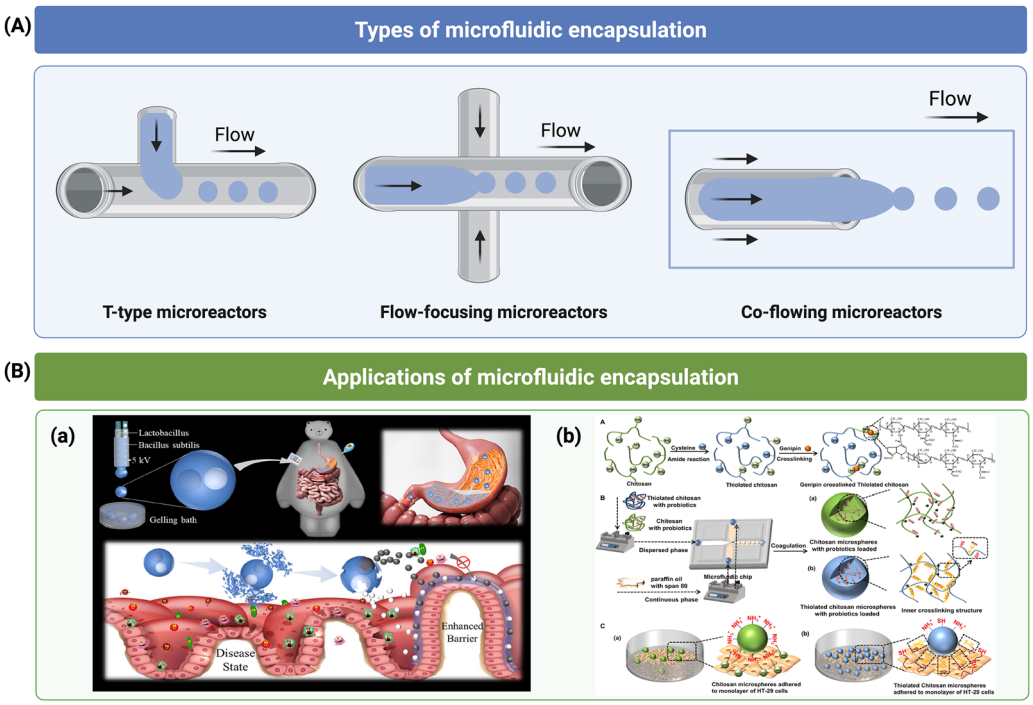

Fig. 5 (A) Schematic illustration of microfluidic encapsulation systems, including T-type, flow-focusing, and co-flowing microreactors. (B) Applications of microfluidic technology in probiotic encapsulation. (a) Electrostatic microfluidic device forming dual-core microcapsules by separately introducing *Lactobacillus* and *Bacillus subtilis*, followed by gelation and gastrointestinal delivery, reproduced from ref. 71 with permission from American Chemical Society, copyright 2020. (b) Polymethyl methacrylate (PMMA) microfluidic chip used to prepare monodisperse thiolated chitosan microspheres encapsulating *Bifidobacterium* FL-276.1. The droplets were crosslinked *in situ* using sodium tripolyphosphate solution, and the resulting microspheres exhibited mucoadhesive behavior in the intestinal tract, reproduced from ref. 74 with permission from Elsevier, copyright 2024.

3.2. 3D printing technology

3D printing technology (also known as additive manufacturing) is an automated manufacturing process that constructs three-dimensional structures layer by layer through computer-aided design and numerical control systems, demonstrating significant potential in customized food design, personalized nutrition, and functional ingredient delivery system development.[120–122] To accommodate different material properties and forming requirements, food 3D printing includes four main printing methods (Fig. 6A): extrusion-based printing (EBP), which relies on a pressure gradient to extrude paste-like or molten materials layer by layer and is suitable for soft materials such as chocolate, dough, and fruit or vegetable purees.[123] Selective sintering utilizes laser or hot air to fuse powdered materials such as sugar, cocoa powder, and starch. It does not require support structures and offers relatively good mechanical properties. However, it involves high-temperature conditions that may not be suitable for heat-sensitive bioactive substances.[124,125] Inkjet printing deposits low-viscosity liquids precisely onto substrates through thermal or piezoelectric mechanisms. It features high precision and fast printing speed, but is limited by the viscosity requirements of printable materials.[126] Binder jetting selectively sprays a binder solution onto a powder bed to form three-dimensional structures. This method can fabricate complex geometries with varied textures at relatively low cost, though the range of usable materials is comparatively narrow.[127,128] These technologies offer the advantage of precisely controlling the internal structure and component distribution of food products, enabling the fabrication of complex geometries and functional designs that are difficult to achieve using traditional manufacturing methods. They are particularly suitable for the protective encapsulation of bioactive components such as probiotics.

Probiotics, as living microorganisms, have their survival and functional maintenance in the 3D printing environment comprehensively affected by material formulation, processing conditions, and post-processing procedures.[128] The design of bio-inks is a core element and must avoid organic solvents and toxic substances. Common carriers include aqueous solutions, milk, or natural polysaccharides and proteins, with pH maintained within a probiotic-tolerant range to preserve cell viability.[78,79,85,130] Printing parameters directly affect probiotic viability, including nozzle diameter, extrusion rate, printing temperature, and layer height.[80] For example, Liu *et al.* encapsulated *Bifidobacterium animalis* BB-12 in 3D-printed mashed potatoes and evaluated the effects of process parameters on probiotic survival by controlling nozzle diameter (0.6, 1.0, 1.4 mm) and printing temperature (25, 35, 45, 55 °C).[81] When the nozzle diameter was 0.6 mm, the viability decreased from 9.93 log CFU g^{-1} in the control to 9.74 log CFU g^{-1}, while 1.0 mm and 1.4 mm nozzles had no significant impact (recording 9.85 and 9.89 log CFU g^{-1}, respectively). Regarding temperature, viability remained stable from 25 to 45 °C but dropped sharply to 7.99 log CFU g^{-1} after 45 min at 55 °C,

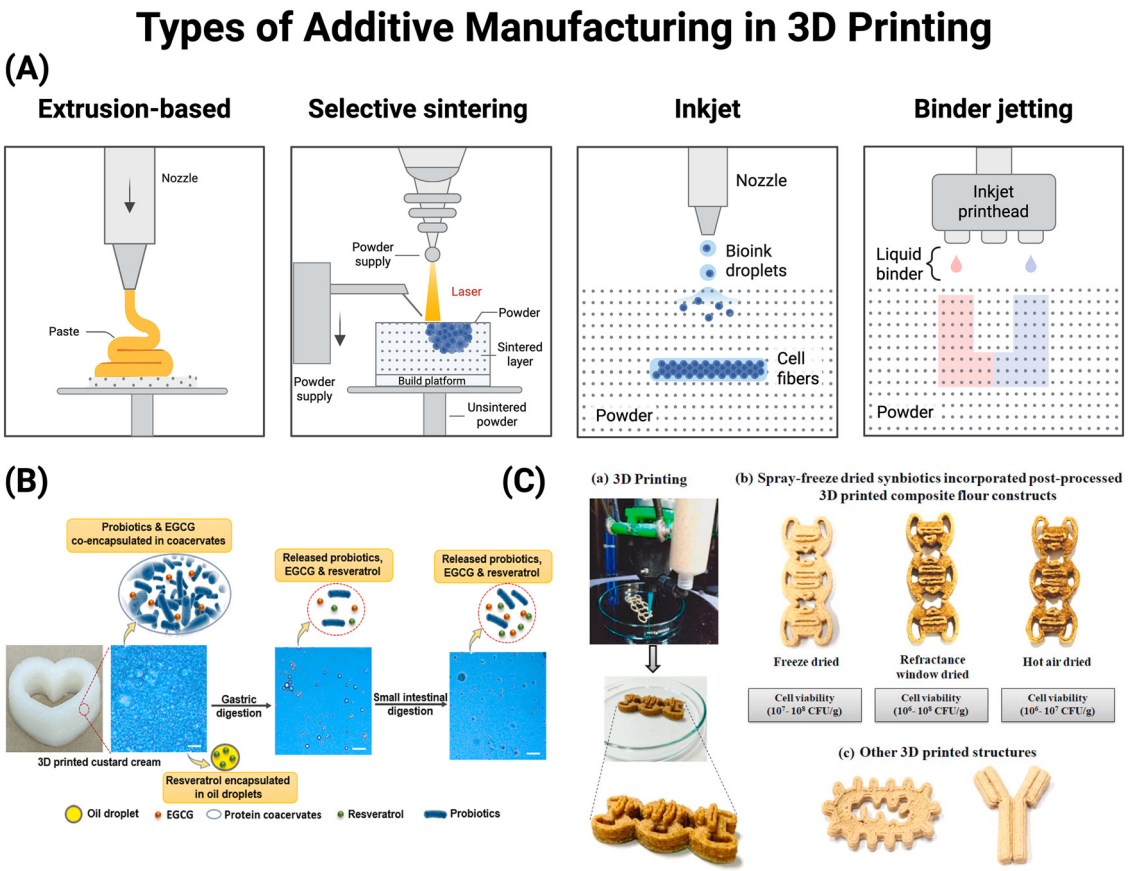

Fig. 6 (A) Schematic illustration of four main types of additive manufacturing in food 3D printing: extrusion-based printing, selective laser sintering, inkjet printing, and binder jetting (created with BioRender). (B) Custard cream prepared by extrusion-based 3D printing with co-encapsulation of probiotics, epigallocatechin gallate (EGCG), and resveratrol, reproduced from ref. 82 with permission from Elsevier, copyright 2023. (C) Comparison of different drying methods applied to 3D printed *Lactiplantibacillus plantarum* (NCIM 2083), including freeze-drying, refractance window drying, and hot air drying, reproduced from ref. 129 with permission from Elsevier, copyright 2021.

representing a 20.6% reduction. The use of a smaller nozzle introduced higher shear stress and longer printing time (1304 s for 0.6 mm *vs.* 273 s for 1.4 mm), increasing oxygen exposure to the anaerobic *Bifidobacterium*, while high temperature led to protein denaturation and membrane damage. In another study, Xu *et al.* encapsulated *Bifidobacterium* using a Pickering emulsion gel stabilized by tea protein and xanthan gum and found that probiotic viability remained within 8.08–8.11 log CFU g^{-1} across nozzle diameters of 0.41, 0.84, and 1.20 mm.[131] Printing at 45 °C and 55 °C had no significant effect on viability, whereas at 65 °C viability decreased from 8.07 to 6.95 log CFU g^{-1}. In the synergistic application of functional components, Cai *et al.* used extrusion-based 3D printing (0.8 mm nozzle, 100 mm s^{-1} printing speed) to co-encapsulate probiotics with EGCG and resveratrol in custard cream (Fig. 6B).[82] After 16 days of storage, probiotic loss was less than 0.2 log CFU g^{-1}, and compared with free probiotics, the co-encapsulated formulation showed reduced loss by 1.58 and 0.5 log CFU g^{-1} under thermal treatments at 63 °C and 75 °C, respectively. Following simulated gastrointestinal digestion, the loss ranged from 0.85 to 1.52 log CFU g^{-1}, and the release rates of EGCG and resveratrol reached 68.10% and 71.69%, respectively. The antioxidant properties of polyphenols helped alleviate oxidative stress in the matrix and reduced cell death. Post-processing conditions are equally important, including drying methods, storage temperature, humidity control, and packaging methods, all of which collectively determine the viability and stability of probiotics in the final product.[132] Yoha *et al.* investigated four post-processing methods (freeze-drying, refractance window drying, hot air drying, and microwave drying) on 3D-printed *Lactiplantibacillus plantarum* (NCIM 2083) (Fig. 6C).[129] Freeze-drying yielded the highest survival rate (>90%) in synbiotic products produced *via* both direct freeze-drying and spray-freeze-drying. The combination of spray-freeze-drying encapsulation and freeze-drying post-treatment exhibited the best performance under simulated gastrointestinal conditions, with a survival rate of 79% and a viable cell count of 6.43 ± 0.17 $\log_{10}$ CFU mL^{-1}. After 35 days of storage, the survival rate remained at 96–98%.

Overall, 3D printing offers a highly controllable approach for personalized delivery of probiotics and functional components, allowing precise control of spatial distribution and dosage through structural design. Nevertheless, this technology still faces challenges such as slow printing speed, limited available bio-ink systems, and potential damage to probiotic viability

caused by shear stress and temperature fluctuations.[83–85] Future progress depends on the development of novel food-grade printable materials compatible with diverse probiotics and the optimization of process parameters to improve efficiency and stability, thereby advancing its application in personalized nutrition and functional food development.

3.3. LbL encapsulation

LbL encapsulation is a method based on the construction of polyelectrolyte multilayer films. Its core principle relies on the electrostatic interactions between oppositely charged macromolecular materials on the surface of a substrate, leading to the stepwise deposition of a multilayer structure with controllable thickness.[86,87,133] During the LbL encapsulation process, polycation and polyanion solutions alternately interact with the substrate. Due to the charge complementarity-driven adsorption, each deposition results in a reversal of surface charge, which in turn facilitates the binding of the next layer.[133,134] This self-limiting adsorption mechanism ensures precise control of each layer thickness, typically within the nanometer range.[135] The driving forces for LbL encapsulation primarily include electrostatic interactions, while also involving hydrogen bonding, van der Waals forces, hydrophobic interactions, and other intermolecular forces, providing considerable flexibility in material selection.[7] By controlling key parameters such as ionic strength, pH, polymer concentration, and deposition time, the thickness, porosity, mechanical strength, and permeability of the final multilayer structure can be precisely regulated.[86,88,136]

LbL systems are typically classified as unigenric or polygenric, depending on the composition of the encapsulation materials.[7] Unigenric encapsulation materials refer to systems in which all encapsulating components belong to the same material category (*e.g.*, pure polysaccharides, proteins, or lipids), while polygenric encapsulation materials involve combinations of different material types to achieve synergistic effects.[137] Unigenric systems possess advantages such as simplified processing, predictable interactions, and clear release mechanisms, but may have limitations in functional diversity and protective capacity. For example, Luan *et al.* employed chitosan/sodium alginate multilayer coatings to encapsulate *Lactiplantibacillus plantarum* subsp. *plantarum* CICC 6240.[89] In this system, nanocellulose served as the core matrix, and positively charged chitosan and negatively charged alginate were alternately deposited *via* LbL encapsulation to form a multilayer protective structure. In SGF (pH 2.0), the multilayer microcapsules (CNFM-CHC-ALG) maintained a survival rate of 80.74% after 2 hours, and 85.61% under bile salt conditions, whereas the unencapsulated strains were completely inactivated. Anselmo *et al.* used the same chitosan/sodium alginate system to encapsulate *Bacillus coagulans*, and the bilayer $(CHI/ALG)_2$ structure significantly enhanced protection of the strain.[138] Free bacteria exposed to SGF for 2 hours were completely inactivated, whereas the encapsulated bacteria showed a reduction of less than 1 log CFU mL^{-1}. Under 4% bile salt treatment, free bacteria exhibited a $\sim$6 log CFU mL^{-1} reduction, while encapsulated bacteria lost less than 2 log CFU mL^{-1}. *In vivo* experiments demonstrated that the encapsulated strain produced a sixfold higher survival signal in the small intestine compared to the free strain. In contrast, polygenic systems combine different types of materials to overcome the inherent limitations of single-type materials, enabling improved environmental adaptability and broader functional regulation.[139] Polysaccharide materials can form stable network structures and exhibit good pH responsiveness,[140,141] protein materials possess film-forming properties and biological activity, as well as natural emulsification characteristics and biocompatibility,[142–144] while lipid materials enhance protection of lipophilic components through hydrophobic interactions.[145] Li *et al.* constructed polygeneric encapsulation of *Lactiplantibacillus plantarum* using whey protein isolate fibrils (WPIF) with carrageenan (CG), hyaluronic acid (HA), and inulin, validating the effectiveness of this strategy (Fig. 7A).[91] In this system, the zeta potential of *Lactiplantibacillus plantarum* shifted from −32.15 mV to +15.55 mV after the first layer of WPIF, followed by alternating positive and negative zeta potential changes, confirming successful LbL multilayer encapsulation. Compared to the 64.71% freeze-drying survival rate of unencapsulated strains, the survival rate of WPIF/CG 1.5-layer encapsulated strains significantly increased to 90.16%, with a survival rate of 84.34% after 4 hours under acidic conditions (pH 3.0), and over 60% activity was retained after simulated gastrointestinal digestion. Zhu *et al.* designed a polygeneric LbL encapsulation system for *Lactiplantibacillus plantarum* B2 using chitosan (CHI), sodium alginate (SA), and mucin (Fig. 7B).[146] The multilayer structure was assembled *via* electrostatic interactions, with SA and mucin used as outer layers to enhance protection against gastric acid, bile salts, and oxidative stress. Both *in vitro* and *in vivo* results indicated improved colon adhesion and probiotic retention in composite encapsulation groups. The study by Wang *et al.* further confirmed the synergistic advantages of polygenric materials.[92] Using a gelatin-hyaluronic acid multilayer system to encapsulate *Lacticaseibacillus rhamnosus* 6133 (Lr-6133), four-layer microcapsules showed only 0.99 log CFU mL^{-1} cell loss under simulated gastrointestinal conditions, far superior to the 3 log CFU mL^{-1} loss of single-layer sodium alginate microcapsules, with encapsulation efficiency reaching 78–92%.

The choice between unigenric and polygenric methods depends on multiple factors, including material compatibility, properties of the target compound, processing conditions, and desired release characteristics. The thickness of each polyelectrolyte bilayer typically ranges from 1 to 10 nm, depending on the molecular weight, conformation, and deposition parameters of the selected materials. Under optimized conditions, the encapsulation efficiency of LbL systems usually reaches 80-95%, but actual values vary depending on material combinations and process parameters.[7,147] However, this technology still faces challenges in the selection of biocompatibility encapsulation materials and maintaining long-term stability of multilayer film structures.[7,93] Future focus should be on screening and optimizing safe, food-grade materials, as well as enhancing structural stability of multilayer films through strategies such as crosslinking degree regulation and stabilizer addition, to

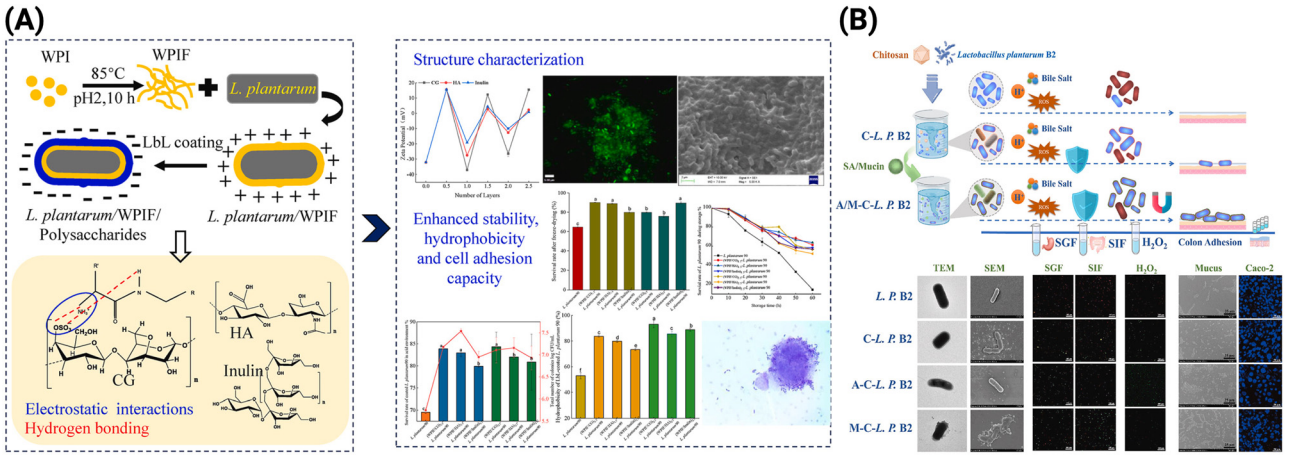

Fig. 7 (A) LbL encapsulation of *Lactiplantibacillus plantarum* using whey protein isolate fibrils (WPIF), carrageenan (CG), hyaluronic acid (HA), and inulin, reproduced from ref. 91 with permission from Elsevier, copyright 2025. (B) A polygeneric LbL encapsulation system was constructed for *Lactiplantibacillus plantarum* B2 using chitosan (CHI), sodium alginate (SA), and mucin. The figure shows probiotic viability under simulated gastric fluid (SGF), intestinal fluid (SIF), and hydrogen peroxide (H_2O_2), as well as adhesion performance on a simulated intestinal mucus layer and Caco-2 cell monolayers. The microstructure of the encapsulated probiotics was characterized by transmission electron microscopy (TEM) and scanning electron microscopy (SEM), reproduced from ref. 146 with permission from Elsevier, copyright 2025.

further improve the practicality and reliability of LbL encapsulation technology.

3.4. Electrospinning/electrospraying technology

Electrospinning and electrospraying are both based on the principles of electrohydrodynamics, where a high-voltage electrostatic field is applied to transform a polymer solution into a jet that ultimately solidifies into a carrier. When the polymer solution at the needle tip is subjected to an electric field, surface tension and Coulombic repulsion reach equilibrium to form a Taylor cone.[8] After the jet ejects from the cone tip, its morphological evolution depends on the rheological properties of the solution: chain entanglement effects present in high-viscosity, high-molecular-weight polymer solutions maintain jet continuity, forming fibrous products (electrospinning); while jets of low-viscosity solutions undergo Rayleigh instability fracture dominated by surface tension, producing spherical particles (electrospraying).[94,148,149] Key process parameters for both technologies include applied voltage, nozzle-to-collector distance, solution flow rate, and environmental humidity, which directly affect the microstructure of carriers and the encapsulation efficiency of probiotics.[44,102,150]

Electrospinning technology can be divided into three main forms based on nozzle configuration and solution systems: uniaxial electrospinning, coaxial electrospinning, and emulsion electrospinning (Fig. 8). Among them, uniaxial and coaxial electrospinning are most widely used in probiotic encapsulation due to their operational simplicity and avoidance of potential surfactants toxicity to probiotics.[96] Uniaxial electrospinning uses a single nozzle to mix probiotics with polymer solution for spinning, forming probiotic-loaded fiber structures driven by high-voltage electrostatic fields. Probiotics are dispersed within the fiber network through physical encapsulation, avoiding complex multilayer preparation processes. During the spinning process, the probiotic-containing polymer solution forms a Taylor cone at the needle tip and is stretched into continuous fibers, with the solvent rapidly evaporating in flight to solidify the fibers. The rheological properties of the solution have a decisive influence on fiber formation quality, with appropriate viscosity maintaining jet continuity and preventing droplet breakage, while conductivity and surface tension affect Taylor cone stability and final fiber morphology. Applied voltage (typically 10–30 kV) is also critical for obtaining ideal fiber structures.[148,151] However, the rapid solvent evaporation may pose risks to probiotic viability, as abrupt osmotic changes can damage cell membranes.[95] For example, Xu *et al.* successfully encapsulated *Lacticaseibacillus rhamnosus* 1.0320 using pectin/polyvinyl alcohol (PVA/PEC) as the matrix through uniaxial electrospinning technology, and investigated the effects of different ratios on nanofiber morphology and bacterial survival rates.[97] At a PVA : PEC ratio of 9 : 1, the obtained fibers showed uniform, bead-free continuous structures with an average diameter of approximately 150 nm, and the encapsulated bacteria maintained a survival rate of 84.63% after 21 days of storage at 4 °C. In another study, Wei *et al.* utilized a mixed system of PVA and silk fibroin (SF) to prepare *Lactiplantibacillus plantarum*-loaded nanofibers, significantly improving fiber continuity and bacterial survival rates by optimizing SF content.[152] After 2 hours of treatment in artificial gastric juice (pH ≈ 1.2), the optical density (OD) of the cultures in PVA/SF nanofibers reached 2.75, far higher than that of the unencapsulated group (OD ≈ 0.17), indicating that PVA/SF nanofibers provided an effective protective barrier for probiotics. Coaxial electrospinning technology constructs core–shell structures through dual concentric nozzles, with the inner aqueous phase providing gentle carrier conditions for probiotics and the outer polymer shell forming a protective barrier.[99] Compared to uniaxial electrospinning, coaxial configurations prevent probiotics from being directly exposed at the

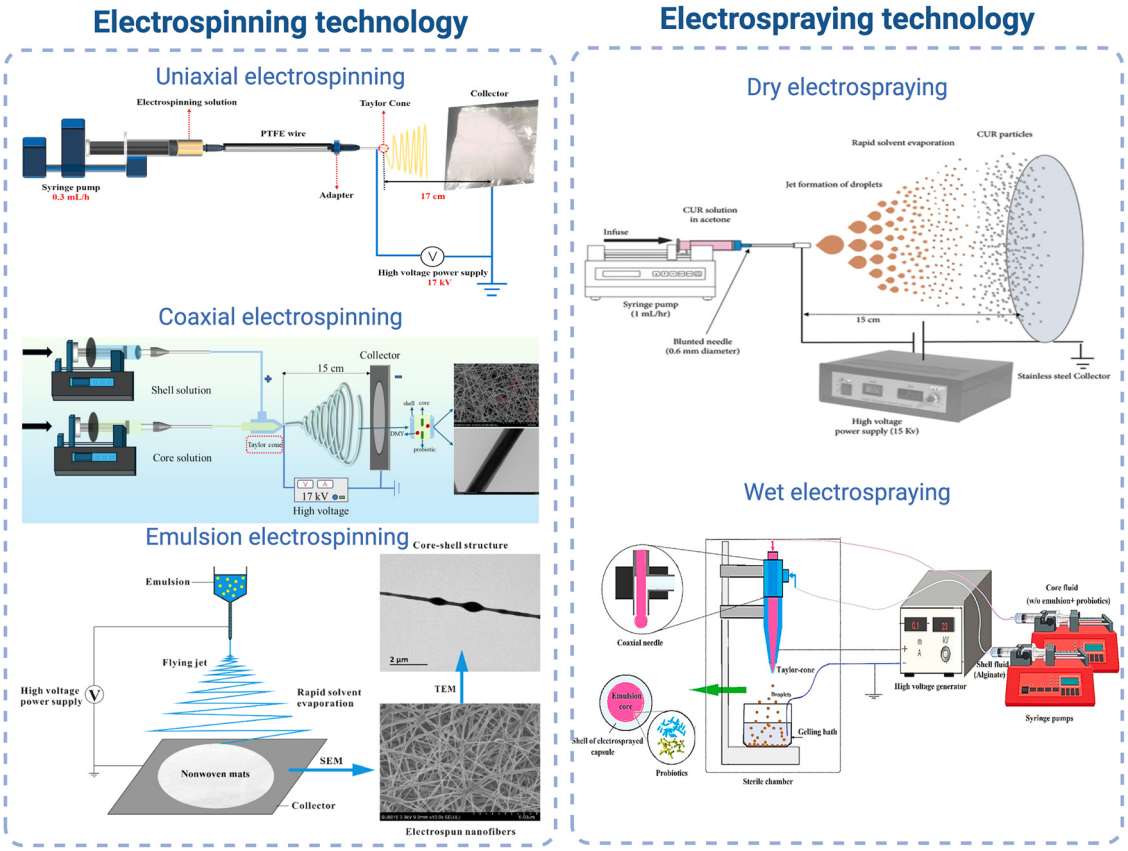

Fig. 8 Overview of electrohydrodynamic techniques, including electrospinning (uniaxial, reproduced from ref. 97 with permission from Elsevier, copyright 2022; coaxial, reproduced from ref. 99 with permission from Elsevier, copyright 2025; and emulsion, reproduced from ref. 154 with permission from Elsevier, copyright 2018) and electrospraying (dry, reproduced from ref. 155 with permission from Wiley, copyright 2024, CC BY 4.0 license; and wet, reproduced from ref. 107 with permission from Elsevier, copyright 2024).

fiber surface, resulting in a more complete encapsulation. The independent control of the core phase allows the use of aqueous solvents compatible with probiotics, avoiding damage to cell membranes by organic solvents.[153] Xu *et al.* constructed core–shell fiber structures loaded with *Lacticaseibacillus rhamnosus* 1.0320 using Eudragit S100 (ES100) as the shell material and poly(vinyl alcohol)/pectin (PVA/PEC) as the core material *via* coaxial electrospinning technology.[98] This study used a 14% ES100 solution as the shell solution, with a shell-to-core flow rate ratio of 4 : 1 (1.6 mL h^{-1} : 0.4 mL h^{-1}), preparing fibers at an applied voltage of 15 kV. The ES100 shell remained stable in gastric fluid (pH $<$ 7) and released probiotics in the colonic environment (pH $>$ 7). The survival rate of encapsulated *Lacticaseibacillus rhamnosus* 1.0320 in SGF and SIF reached 90.07% and 91.96%, respectively, with 81.40% retained after sequential gastrointestinal digestion. In contrast, PVA/PEC fibers prepared by uniaxial electrospinning showed limited protection due to their water solubility and poor resistance to gastrointestinal fluids. Tan *et al.* constructed a polyvinyl alcohol-fucoidan@ethyl cellulose (PVOH-FUC@EC) coaxial electrospinning system for co-encapsulation of *Lactiplantibacillus plantarum* 69-2 and dihydromyricetin.[99] By adjusting the shell-to-core flow rate ratio (1 : 1, 1 : 1.5, and 1 : 2), shell thickness increased from 64.94 ± 2.35 nm to 96.76 ± 3.62 nm. After sequential gastrointestinal digestion, the survival rate of *Lactiplantibacillus plantarum* 69-2 encapsulated in PVOH-FUC@EC core–shell fibers was 81.97–88.87%, compared to 62.79% for uniaxial PVOH-FUC fibers. Additionally, the core–shell structure enhanced the antioxidant properties of the fibers, with ABTS+ and DPPH radical scavenging rates reaching 75.35% and 65.73%, respectively. Emulsion electrospinning technology disperses probiotic suspensions in polymer solutions through surfactants to form stable water-in-oil (W/O) or oil-in-water (O/W) emulsion systems. This technology utilizes emulsion droplets as microreactors to encapsulate probiotics, forming composite fiber structures with core–shell characteristics as the solvent evaporates during spinning.[44] Although this technology can achieve protective effects similar to coaxial electrospinning through emulsification, avoiding direct contact between cells and the external environment, the introduction of surfactants may compromise probiotic cell-membrane integrity, and the preparation of emulsion systems requires precise control of emulsification conditions, surfactant concentration, and oil–water ratios, resulting in high process complexity and relatively limited application in probiotic encapsulation.[8,100]

Electrospraying technology comprises dry and wet collection modes, which are suitable for different encapsulation requirements (Fig. 8). Dry electrospraying deposits solidified particles onto a solid collector. When polymer concentration is low, the

jet undergoes Rayleigh instability breakup dominated by surface tension, producing spherical particles. Process parameters such as polymer concentration, solution viscosity, applied voltage, and flow rate influence particle formation.[101,107,148] Increasing voltage typically reduces particle size and improves the uniformity of particle size distribution, while increasing flow rate increases particle diameter. This technology has the advantages of simple operation, high encapsulation efficiency, and convenient storage of products. However, osmotic pressure shock during rapid solvent evaporation is the main factor affecting probiotic survival.[102] Moayyedi *et al.* employed three carrier systems, including whey protein isolate (WPI), whey protein isolate + inulin (WPI + IN), and whey protein isolate + inulin + Persian gum (WPI + IN + PG) to comparatively study the effects of electrospraying, freeze-drying, and spray-drying technologies for preparing microcapsules loaded with *Lacticaseibacillus rhamnosus* ATCC 7469.[104] Under processing conditions of 14 kV voltage, a flow rate of 0.7 mL h^{-1}, and a needle-to-collector distance of 7 cm, the microcapsules produced by electrospraying exhibited uniform spherical morphology with intact surfaces and particle sizes ranging from 359 to 596 nm. In comparison, freeze-dried products showed irregular shapes with surface indentations (341–364 nm), and spray-dried products exhibited wrinkled surfaces (295.7–353 nm). However, cell damage analysis indicated that cells treated by electrospraying were the most sensitive to 4% NaCl stress. The cell damage rates were 53.3%, 48.55%, and 43.66% for the WPI, WPI + IN, and WPI + IN + PG groups, respectively, all of which were higher than those observed in the corresponding freeze-dried (47.65%, 39.87%, 39.50%) and spray-dried (44.74%, 39.77%, 40.74%) groups. The high-voltage electric field and osmotic pressure changes were identified as the main causes of this phenomenon.

Wet electrospraying sprays probiotic-containing polymer droplets into a crosslinking agent solution, instantly forming hydrogel microspheres through ionic crosslinking and avoiding solvent evaporation damage to cells. This technology mainly relies on the specific binding of divalent metal ions (such as Ca^{2+}) with polysaccharide molecules to form stable three-dimensional network structures.[105] The polymer concentration directly determines the microsphere diameter, with higher concentrations resulting in larger microspheres. However, excessively high concentrations may cause nozzle clogging due to increased viscosity. The concentration of the crosslinking agent is also important. Higher crosslinker concentrations can accelerate the crosslinking process and reduce microsphere size. In addition, the applied voltage and the distance between the nozzle and the crosslinking bath affect the final properties of the resulting microspheres.[156] The advantages of wet electrospraying include mild processing conditions, the avoidance of organic solvents, and extremely high encapsulation efficiency, but it is limited to specific material systems capable of ionic crosslinking.[106] Farahmand *et al.* employed coaxial wet electrospraying to encapsulate *Bifidobacterium animalis* subsp. *lactis* and *Lactiplantibacillus plantarum* PTCC 1896 within a W/O emulsion-calcium alginate system. *Lactiplantibacillus plantarum* exhibited similar encapsulation survival rates following wet electrospraying and freeze-drying (92.06% *vs.* 92.16%), whereas *Bifidobacterium animalis* subsp. *lactis* showed rates of 86.46% and 97.25%, respectively.[107] After sequential gastrointestinal digestion, the viable counts of both strains encapsulated by wet electrospraying remained above 10^8 CFU g^{-1}, while freeze-dried samples decreased to approximately 10^7 CFU g^{-1}. At −18 °C storage conditions, wet electrospraying encapsulated samples maintained viability for more than 5 months, superior to the 4 months of freeze-drying. Additionally, Zaeim *et al.* prepared calcium alginate/chitosan microcapsules containing *Lactiplantibacillus plantarum* ATCC 8014 and *Bifidobacterium animalis* subsp. *lactis* through wet electrospraying technology, and formed composite encapsulation systems by adding inulin or resistant starch.[49] This technology achieved 98% probiotic encapsulation efficiency, with *Bifidobacterium animalis* subsp. *lactis* showed the highest survival rate after gastrointestinal treatment, reducing by only 1.6 log cycles.

Electrohydrodynamic methods, as emerging encapsulation strategies, have been continuously studied in probiotic carrier construction, but their practical application is still limited by material properties and process stability. During electrospinning, food-grade materials often require the addition of synthetic polymers or the use of organic solvents due to insufficient spinnability, which may lead to solvent residue issues.[108] Furthermore, the nonlinear relationship between polymer concentration and particle morphology during electrospraying makes process optimization highly challenging, especially in multi-component systems where coupling effects between components further increase the complexity of parameter control.[106] Future research can focus on the following directions: (1) developing biocompatible electrospinning solvent systems suitable for probiotic encapsulation; (2) establishing machine learning-based prediction models for process parameters to enable quantitative design of carrier properties; (3) integrating online monitoring technologies to achieve closed-loop quality control through real-time detection of jet behavior and particle morphology.

4. Functional evaluation

Functional evaluation is essential for verifying the survival, metabolic activity, and host interactions of probiotics in the gastrointestinal environment. Commonly used approaches include *ex vivo* simulated gastrointestinal models (static models, dynamic models such as TNO Intestinal Model (TIM) and Simulator of the Human Intestinal Microbial Ecosystems (SHIME), and organoids/*ex vivo* tissues) and *in vivo* tracking and analysis (real-time imaging technology, metabolomics tracking, and microbiome–host interaction analysis). These methods collectively provide insights at various levels into the functionality and regulatory mechanisms of probiotics. Fig. 9 summarizes the basic classifications and features of these approaches, presenting a comprehensive framework that spans from *ex vivo* to *in vivo*, from static to dynamic, and ultimately to multi-omics integration.

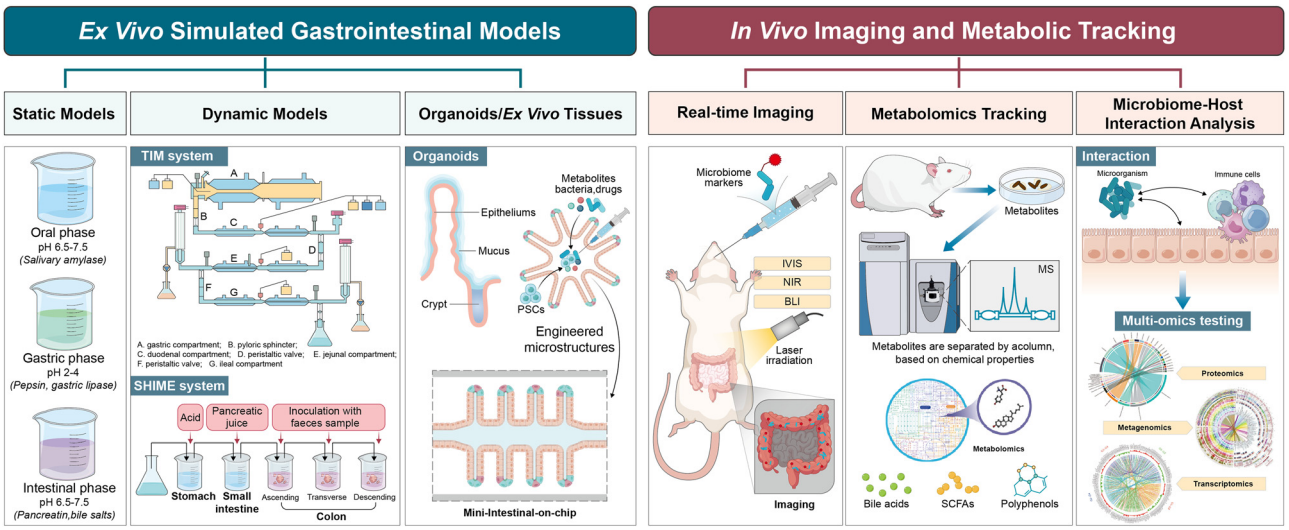

Fig. 9 A summary and classification of the methods for evaluating the biological functions of probiotics. This figure systematically reviews (from left to right) *ex vivo* simulated gastrointestinal models and *in vivo* imaging and metabolic tracking techniques for assessing the health effects and mechanisms of action of probiotics on the gastrointestinal tract, spanning from *ex vivo* to *in vivo*, and from micro to macro. The *ex vivo* simulated gastrointestinal models comprise static models (simulating the oral, gastric, and intestinal environments), dynamic models (such as TIM and SHIME systems), and organoids/*ex vivo* tissues (including mini-intestines and stem cells). In addition to near-infrared imaging, *in vivo* imaging, and bioluminescence imaging methods, metabolic tracking techniques such as mass spectrometry imaging and isotope tracing, as well as multi-omics integration (proteomics, metagenomics, transcriptomics) analysis of microbiome-host interactions, can be used for precise verification and data integration to deeply explore the functional mechanisms and regulatory strategies of probiotics in gastrointestinal health and disease.

4.1. *Ex vivo* simulated gastrointestinal models

4.1.1. Static models. *In vitro* methods to simulate gastrointestinal digestion have been widely used in many areas of food and nutritional science, particularly to study the gastrointestinal behavior of foods or drugs. *In vitro* gastrointestinal models are mainly divided into three categories: static models, dynamic models, and organoid/*ex vivo* tissue models, among which static models are the simplest and easiest to implement, and most of them consist of three stages: oral, gastrointestinal, and intestinal. These traditional single-chamber models simulated enzyme, electrolyte, pH, temperature, and bile salt conditions to evaluate the stability, release characteristics, and functional synergy of the probiotic-embedded delivery system during digestion.[157–159] Simulation of the oral digestive environment can be achieved through salivary amylase, saliva from laboratory volunteers, or even using saliva that has been actually chewed by volunteers.[160] The gastric phase is usually in a constant acidic environment (pH 2–2.5) and contains a mixture of digestive enzymes, under which food samples are incubated for 2 hours to mimic the gastric digestion process. Subsequently, the digestion product is transferred to the intestinal phase, which is carried out under constant neutral to weakly alkaline conditions (pH 6.5–7.5) supplemented with corresponding buffers and digestive enzymes for 4–6 hours in a static incubation pattern, thus mimicking the intestinal digestion environment.[161,162] García-Ruiz *et al.* demonstrated that probiotics retain their viability even when exposed to saliva, and that lactic acid bacteria and *Pediococcus* strains still exhibit high resistance (>80%) to 100 mg L^{-1} lysozyme under conditions that mimic those of dilution in saliva. In addition, the survival rate of most wine strains decreased by only a single log unit in the environment of low pH (pH 1.8) and bile salts, indicating that they have good adaptability to gastrointestinal conditions.[163] Kemsawasd *et al.* investigated the viability of immobilized potential probiotics (*Lacticaseibacillus casei* 01 and *Lactobacillus acidophilus* LA 5) during storage in three different types of chocolate (white, milk, and dark chocolate). It was found that after 2 hours of exposure to the simulated gastric environment, the chocolate immobilized with *Lacticaseibacillus casei* 01 had a high survivability.[164] Subsequently, these fixed *Lacticaseibacillus casei* 01 cells, after being incubated in SIF for an additional 4 hours and found that they still maintained a high survival rate. In addition, there is a close symbiotic relationship between the gut microbiota and its host, and a key component of gut homeostasis is the presence of a mucus layer covering the gastrointestinal tract.[165] Different cell lines (*e.g.*, primary or transformed cells) can be used for 2D culture, Caco-2,[166] HT-29,[167] and T84[168] provide a host-like gut microenvironment for cells and can be used to understand bacterial survival and replication. McCright *et al.* successfully simulated the mucus-producing human intestinal epithelial environment by co-culturing Caco-2 and HT-29, and the mucus produced when Caco-2 and HT 29 were co-cultured in 90 : 10 and 80 : 20 ratios had the same mechanical properties as porcine jejunal and ileal mucus.[169] Biocompatible, biodegradable and non-toxic delivery systems, including biopolymer particles, such as alginate beads, are particularly suitable for the food industry to protect probiotics from degradation and deliver as many high-quality probiotics as possible to the target organ.[170–172] Qian *et al.* formulated mock gastric juice (SGF) containing pepsin and then adjusted the pH to 2.0 with 0.1 N HCl.[173] A mock SIF containing bile salts, pancreatic enzyme preparations was

prepared, and the pH was adjusted to 7.5 using 0.1 M $NaHCO_3$. After a mixture of 100 mL of free probiotics or 1.0 g of fresh beads was incubated in 9.0 mL of SGF at 37 °C for 2 hours with shaking, the pH of the system was adjusted to 7.5 and 12 mg mL^{-1} bile extract and 2 mg mL^{-1} pancreatic enzyme preparation were added. The results showed that sodium alginate microspheres had no protective effect for either free probiotics or encapsulated probiotics due to combined exposure to calcium ions and harsh gastrointestinal conditions (*e.g.*, hydrogen ions, bile salts, and digestive enzymes) in GIT under this static model. However, the viability of probiotics encapsulated in calcium alginate and insect skin polysaccharide/emulsion microgel was still higher than 7.0 log CFU viable cells per g after gastrointestinal digestion.

Static models can be used to quantitatively analyze the yield of key metabolites in probiotics, such as SCFAs. Raval *et al.* showed that all SCFAs except *Bacillus* spp. had higher yields under anaerobic conditions, especially under aerobic conditions, which seemed to be more beneficial for increasing their acetic acid production.[174] More interestingly, they found significantly higher yields of butyric acid and propionic acid when synbiotic was administered, compared to the administration of probiotics or prebiotics alone. In addition, the abundance of lactobacilli and bifidobacteria with a synbiotic mixture was significantly higher than that of probiotics or prebiotics alone. Navez *et al.* followed the validation method of the SHIME sample (a static model adapted from the SHIME model) for SCFAs analysis of four phages isolated from the intestinal microbiota of piglets, and the results showed that the phages did not significantly inhibit bacterial colonization or homeostasis of the model over time.[175] In addition, static models can be used to assess the bioavailability of ingested substances. Sharma *et al.* utilized this method to determine the bioavailability and bioactivity of capsule powders containing polyphenol extracts and probiotics.[176] The results showed that TPC decreased from 8.2 mg GAE per g (non-digested) to 5.2 mg GAE per g (intestinal digested) due to the sensitivity of phenolic compounds to GIT conditions, and the DPPH and ABTS scavenging activity decreased by 51.1% and 58.5%, respectively. In contrast, Kashyap *et al.* mentioned that under digestive conditions from the stomach to the intestines, the increase in polyphenol content was due to the release of bound polyphenols by the action of digestive enzymes.[177] Using standard experimental equipment, *in vitro* simulated gastrointestinal models have become the standard method for simulating food digestion in both academic and industrial settings. The static model can not only be used to evaluate the effect of digestive conditions on the viability and stability of probiotics, playing an important role in the bioavailability and functionality of nutrients and active substances; but also can be used to quickly screen the formulation of foods, optimize their processes and preliminary mechanism research, and have the advantages of low cost and short cycle.[178] However, static models cannot replicate some of the important dynamic processes that occur during gastrointestinal digestion, including host-microbial interactions. Therefore, researchers use more convincing dynamic models (such as TIM systems and SHIME) to simulate the real physiological environment, conduct comprehensive efficacy evaluation, and provide clinical (pre-)validation.[179]

4.1.2. Dynamic models. The human gastrointestinal tract is a highly dynamic system that includes mechanical processes, physical digestion, and gradual changes in gastrointestinal conditions in the body, such as peristaltic mixing, and dynamic changes in pH.[180] The TIM (TNO Gastro-Intestinal Model) is a multi-compartment *in vitro* model created by Havenaar and Minekus. The TIM-1 model comprises four computer-controlled compartments simulating the stomach, duodenum, jejunum, and ileum, while the TIM-2 model simulates the large intestine. By integrating a number of key physiological parameters, the dynamic events occurring in the gastrointestinal tract lumen of humans and monogastric animals can be accurately simulated. In particular, the model reproduces key dynamic processes such as the acidic environment of the stomach and the gradient of bile salt concentrations in the small intestine, which determine the survival of probiotics.[181–183] Surono *et al.* evaluated the survival of free or microencapsulated probiotics (*Lactiplantibacillus plantarum* IS-10506 or *Enterococcus faecium* IS-27526) resuspended in milk using the TIM-1 system.[184] The average survival rates of free *Enterococcus faecium* and *Lactiplantibacillus plantarum* were 15.0% and 18.5%, respectively. However, microencapsulation markedly improved the survival of *Lactiplantibacillus plantarum* (84.5%) but not *Enterococcus faecium* (15.7%) TIM system's highly biomimetic properties (*e.g.*, gastric emptying rate, timing of digestive juice secretion, and other parameters are highly consistent with human physiology) make it an ideal tool for studying the behavior of probiotics under near-physiological conditions.[185] Marteau *et al.* measured the survival of individual strains of *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, and *Streptococcus acidophilus* under physiological conditions and compared them with data obtained from humans, and found no significant differences between *in vitro* and *in vivo* data.[186] In addition, acid-sensitive strains (such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) maintained higher viability than most other bacteria in the first 20 to 30 minutes after a meal, when the pH in the stomach was still relatively high (pH > 3.8) reaching the duodenum, a result that underscores the importance of the initial phase of gastric emptying for the delivery of live bacteria into the small intestine.

Although the TIM system can accurately simulate the physicochemical digestion process of the stomach and small intestine, it lacks the integration of the intestinal microbiota. In order to study the interaction between food components and colonic microbiota, the SHIME system developed by Molly *et al.*[187] Expanding upon the TIM framework with a multi-chamber colon reactor, SHIME enabled the first complete *in vitro* simulation of chyme transit from digestion to fermentation. The system simulates the physiological environment of the stomach, duodenum, ascending, transverse and descending colons through five tandem reactors, and also controls the administration of various additives such as bile salts, bile salt hydrolases, probiotics, drugs, and prodrugs.[188] While pioneering the simulation of colonic fermentation, the SHIME system has a relatively simple design and a limited analytical scope, as

it does not model the absorption of metabolites and fluids.[187] In this regard, the SIMGI (Simulator of Gastro-Intestinal) system can further improve the physiological relevance of microbial metabolism prediction by introducing continuous dynamic regulation of stomach-small intestine-colon (such as automatic pH switching and segmented residence time). The system consists of a gastric chamber (simulated peristaltic mixing), a small intestine reactor, and a three-stage continuous colonic reactor (simulated region-specific microbiota). The modules can be operated in tandem (complete simulation of the entire digestion-fermentation process) or independently (separate study of digestion or fermentation stages). This can help assess the homeostatic conditions of the microbial community and intervene in the diet.[181] Russell *et al.* used this system to compare the formation of SCFAs and ammonium under high-energy (microbiota stabilization) and low-energy (dietary intervention) diets. They found that in a two-fold reduction in the average amount of total SCFAs across the three colonic compartments compared to the period of high energy intake.[189] In addition, the transition from a high-energy medium to a low-energy medium resulted in a 2-fold increase in ammonium content in the distal colonic compartment and a significant 6-fold increase in ammonium content in the proximal colonic compartment. This result is compared with *in vivo* data in obese subjects, and it can be assumed that when individuals consume a diet high in protein and reduced total carbohydrates, a significant decrease in SCFA and an increase in proteolysates are observed.

Although the SIMGI system completely simulates the metabolic changes of microbiota along the digestive tract and can flexibly adjust parameters and employ automatic control to better simulate the human gastrointestinal tract, it cannot analyze how these metabolites affect the host intestinal barrier. The ESIN system recreates the complex physiology of the human stomach and small intestine by integrating engineered intestinal epithelial and immune cell co-culture modules, which incorporates features such as pH, delivery time, chyme mixing, digestive secretions, and passive absorption of digestive products, filling this gap, enabling the first *in vitro* model to study the two-way interaction of microbial metabolism and host response.[190]

4.1.3. Organoids/*ex vivo* tissues. To more realistically simulate the dynamic interactions among various components of the gut, different three-dimensional (3D) models have been developed.[191] Organoids are the 3D *ex vivo* model that is closest to *in vivo* conditions, which can be maintained in culture for a long time through repeated passages while maintaining stable genetic and epigenetic characteristics. This makes them a pivotal tool for studying intestinal morphogenesis and homeostatic maintenance.[192] In 2009, Hans Clevers *et al.* generated the first intestinal organoid using mouse intestinal stem cells and terming a ''mini-gut'' or ''gut-like''.[193] Mitrofanova *et al.* integrated organism and organ-on-a-chip technologies to develop an advanced human organ model that is very similar to living tissue.[194] Since then, organoids have rapidly expanded to a variety of human tissues, providing a model platform closer to human physiology for studying organ development, disease mechanisms, and drug screening, such as Spence *et al.* establishing a human version from human adult stem cells (AdSC) or pluripotent stem cells (PSCs) for studying human intestinal development and disease.[195] The barrier properties of the human small intestine are essential for regulating digestion, nutrient absorption, and drug metabolism. However, most of the existing *in vitro* organoid models are limited to the epithelial layer, ignoring the potential contributions of mesenchymal cells (*e.g.*, fibroblasts) and the ECM to epithelial barrier function. The ECM contains soluble factors, nutrients, and oxygen, and the apical basal polarity resembles that of cells *in vivo* tissue, so it can provide biochemical support to cells within intestinal tissue.[195] Wang *et al.* demonstrated for the first time that YL20 can combat *Cronobacter sakazakii*-induced necrotizing enterocolitis by improving intestinal stem cell function and enhancing barrier integrity using an intestinal organoid model and intestinal barrier model of *Cronobacter sakazakii* infection.[196] The results showed that *Ligilactobacillus salivarius* YL20 promote intestinal organoid epithelial cell proliferation, reverse the low levels of Zo-1 and Occludin mRNA in intestinal organoids induced by *Cronobacter sakazakii*, and restore the respective protein levels in *Cronobacter sakazakii*-infected HT-29 cells. In addition, YL20 prevented the decrease of TEER and the corresponding increase in permeability of *Cronobacter sakazakii*-infected Caco-2 monolayer membranes. TEER is a key parameter that reflects epithelial barrier function (monolayer permeability and integrity).[197] Anjum *et al.* evaluated the effect of 12 strains of bacteria from the human milk of mothers who underwent cesarean delivery on intestinal barrier function in the presence and absence of pathogens.[198] In the presence of most lactic acid bacteria strains, the TEER of the T84 cell monolayer was elevated, most pronounced by *Limosilactobacillus reuteri* NPL-88 (34% within 5 hours), which exceeded the effect of the well-known probiotic *Lactobacillus acidophilus* (20%). In addition, the Ussing chamber uses fluorescent probes to support *ex vivo* measurements of mouse and human tissue permeability, as well as electrophysiological measurements, which can better represent the morphological and physiological characteristics of the intestinal wall.[199,200] Although organoid and *ex vivo* tissue models partially compensate for the limitations of static and dynamic models, they still cannot fully reproduce the complex physiological environment of the human body, such as immune regulation, neural signaling, and microbial diversity. Consequently, findings obtained from these advanced models still require ultimate validation through *in vivo* studies.

4.2. *In vivo* imaging and metabolic tracking

4.2.1. Real-time imaging technology. *In vivo* imaging system (IVIS) is an exceptionally sensitive optical imaging platform that facilitates real-time, quantitative monitoring of biological phenomena within living systems.[201,202] Zhou *et al.* successfully developed an innovative technology combining an IVIS imaging system with DIR membrane dye labeling, which can enable continuous monitoring of bacterial infections in a targeted infection model in a rat bone defect infection model.[203] In addition, by applying fluorescently labeled bacteria topically,

IVIS imaging can capture temporal variations in fluorescence signal within the site of infection. This capability enables assessment of the *in vivo* performance of antimicrobial biomaterials. Liu *et al.* designed a colon-targeted drug delivery system (CTDS) to encapsulate *Lactobacillus paracasei*, utilizing the phytotherapeutic and biocompatible properties of Pueraria lobata.[204] The plasmid pNZ8148-mCherry was used to transform free and encapsulated bacteria, and the pH/enzyme response release kinetics were verified by fluorescence imaging, demonstrating that the probiotics were targeted for delivery to the colon with minimal gastric leakage.

Near-infrared bioimaging is also a very common real-time imaging technique. Ji *et al.* labeled the near-infrared-IIb (NIR-IIb, 1500–1700 nm) lanthanide nanomaterials $NaGdF_4$:Yb^{3+}, Er^{3+} @$NaGdF_4$, Nd^{3+} (Er@Nd NPs) to the surface of *Lactobacillus bulgaricus*, and monitored microorganisms in tissues by two-photon excitation (TPE) microscopy and *in vivo* NIR-IIb *in vivo* imaging.[205] The results showed that the NIR-IIb signal of modified Er@Nd NPs was observed in mouse intestinal tissues with high spatiotemporal resolution (SBRs = 6.88, FWHM = 2.653 mm) and good tissue penetration (~7 mm) under 808 nm excitation. In addition, Zhu *et al.* performed surface nano-coating to encapsulate probiotics using a layer-by-layer coating procedure: the first layer is MPN (EcN@PC–Fe) made of proanthocyanidins (PC) and FeIII ions, while the second layer is the HMW-HA layer, which is incorporated onto the first layer under cytocompatible conditions, yielding EcN@PC–Fe/HA.[206] The researchers used IVIS to monitor the fluorescence signal at different time intervals and found that the uncoated EcN-mcherry remained only in the mouse intestine for about 24 hours, while the fluorescence of EcNmCherry@PC-Fe and EcN-mCherry@PC-Fe/HA was detectable even after 120 hours. Semi-quantitative analysis of fluorescence intensity using Living Image software revealed a 3-fold increase in fluorescence intensity in inflamed colon tissue compared to healthy tissues, indicating improved adhesion and retention of encapsulated probiotics at the inflamed site.

Bioluminescence imaging (BLI) is a powerful method for visualizing biological processes and tracking cells. Typically, luciferase can be expressed in target cells to image cellular viability and fused or co-expressed with target proteins to characterize protein–protein interactions and signaling pathways. Fluorescein can be used as a probe to detect target molecules in an intracellular or intercellular environment.[207] NanoLuc, CBRluc, and Fluc have been expressed in various commensal and probiotic bacteria for whole-body tracking of *Lactiplantibacillus plantarum* and *Escherichia coli*, isolation of the digestive tract, and fecal bacterial enumeration and probing.[208] In addition, spectroscopic examination and quantification of the corresponding strains using the two bioluminescence signals allow the simultaneous study of the dynamics and fate of two different bacterial populations. For example, CBRluc and CBGluc produce red and green luminescent lactic acid bacteria in *Lactococcus lactis* and *Lactiplantibacillus plantarum*, enabling two-color bioluminescence detection to be combined with spectral decomposition.[209]

4.2.2. Metabolomics tracking. Mass spectrometry imaging (MSI) is an analytical technique capable of simultaneously detecting hundreds to thousands of chemical substances and preserving their spatial information.[210] Zhang *et al.* applied matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) to simultaneously perform spatial localization and quantification of metabolites at near-single-cell resolution to analyze *in situ* metabolism in small intestinal tissues.[211] Analysis revealed that goblet cells in the sham group exhibited greater glutamine accumulation compared to neighboring intestinal cells, while this accumulation was significantly reduced in the TPN group and further reduced in the starvation group. Wu *et al.* showed that Fnevs, an extracellular vesicle secreted by Fusobacterium nucleatum, carries the active component of the parental bacterium, allowing bacteria to transmit information without direct contact with the host cell.[212] After detecting differential metabolites by liquid chromatography-tandem mass spectrometry, it was found that Fnevs promotes cell proliferation by regulating amino acid biosynthesis and metabolic pathways in CRC cells, such as central carbon metabolism, protein digestion, and cancer uptake. In addition, transcriptome sequencing showed that differentially expressed genes were mostly involved in the positive regulation of tumor cell proliferation. Zhao *et al.* used ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) to determine the content of polyphenols in the pulp of unfermented apples, finding their levels to be 1.41, 1.38 and 1.36 times higher than those in unfermented apple pulp, respectively. After fermentation and digestion, the antibacterial activity and antioxidant capacity of apple pulp were improved.[213] This suggests that the use of probiotics for fermentation of foods favors the bioavailability of bioactive ingredients. Ma *et al.* investigated the effects of different probiotics on the flavor, characteristic volatile components and differential metabolite distribution of fresh cheese using solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) and non-targeted metabolomics techniques. The results showed that the addition of probiotics in cheese promoted curd formation, enhanced texture, and imparted a unique flavor to cheese, improving cheese quality.[214] Qi *et al.* used LC-MS-based non-targeted metabolomics technology to analyze the value-added metabolites measured by *Bacillus subtilis* DC-15 fermented okara at different fermentation times (0 and 48 hours) to establish a metabolic model.[215] Studies have demonstrated that *Bacillus subtilis* DC-15 can accelerate the decomposition of complex macromolecules, thereby improving the nutrition and function of fermented okara, and the differential metabolites primarily consisted of amino acids, dipeptides, fatty acids, small-molecule sugars and vitamins, and most of these differential metabolites are enriched in amino acid metabolism and glucose metabolism pathways. These studies show that the combination of mass spectrometry and non-targeted metabolomics technology can provide a powerful approach for improving the sensory properties of foods and developing functional foods.

Stable isotope tracing technology has also become a powerful tool for elucidating the gut microbial ecosystem due to its

ability to label specific metabolic pathways and molecules, providing high-resolution and quantitative data. Li *et al.* used glucose tolerance assay and stable isotope tracing to verify that the blood glucose clearance rate was significantly accelerated in mice with intestinal microbiota loss, and found that the glucose uptake of brown adipose tissue (BAT) was significantly increased.[216] Wei *et al.* employed stable isotope tracing technology to reveal the mechanism of inulin protecting the liver, and locked the key mechanism axis of "inulin-Parabacteroides distasonis-pentadecanoic acid".[217] Intestinal beneficial bacteria labeled with ^{13}C-inulin, such as *Bacteroides uniformis*, *Bacteroides acidifaciens*, *Parabacteroides distasonis*, *etc.*; At the same time, a series of ^{13}C-labeled metabolites, such as long-chain fatty acids, adenosine, vitamins, *etc.*, were discovered. The team selected the top three labeled bacteria and administered gavage to mice fed a high-fat diet, and the results showed that *Parabacteroides dieldii* had the most significant effect in inhibiting non-alcoholic steatohepatitis. Matthew *et al.* used isotope labeling and antibiotic treatment to investigate the contribution of intestinal microbiota-mediated urea nitrogen recycling in ground squirrels to protein homeostasis during host hibernation.[218] Compared with summer, the expression of urea channel proteins in the intestines of ground squirrels is increased in winter (especially late winter), which promotes the transport of blood urea (derived from host protein catabolism) to the intestinal lumen. In addition, the abundance of urease genes and their carriers (such as *Alistipes*) in the microbiota increased, which promoted the conversion of urea from the intestinal lumen into metabolites such as ammonia and amino acids. In conclusion, the use of metabolomics to study the distribution and metabolic pathways of probiotics is of great significance for the improvement of food sensory characteristics, the development of functional foods, and the prevention and treatment of diseases.

4.2.3. Microbiome–host interaction analysis. Microbiome–host interaction is a complex and multi-layered field of research, involving multiple disciplines such as metagenomics, metabolome, and transcriptome. Pepke *et al.* suggested that the gut microbiota is an epigenetic effector that affects host gene expression. The host is also able to influence the gut microbiota through histone and DNA modifications that affect immune genes, antimicrobial peptides (AMPs) or intestinal barrier function, for example, through non-coding RNAs (ncRNAs) that may affect microbial gene expression.[219] Alenghat *et al.* demonstrated that in a mouse knockout model, the deletion of HDAC3 in IECs exhibits decreased antimicrobial gene expression, loss of intestinal barrier function, altered gut microbiota composition, and an increased abundance of *Proteobacteria*.[220] This suggests that the feedback signals that affect the microbiota involve a coordinated response to the host epigenetic and transcriptional pathways. In addition, a stable gut microbiome is crucial for maintaining host health. In order to explore how prebiotics can improve the systemic stability of the gut microbiome during the supplementation of probiotics. Ma *et al.* employed *Lactiplantibacillus plantarum* HNU082 as a model probiotic and administered galacto-oligosaccharides either continuously or in pulsed doses. Shotgun metagenomic sequencing was used to assess the effects on gut microbiome stability in mice.[221] The results showed that continuous supplementation of galacto-oligosaccharides under competitive conditions could effectively promote the stable colonization of probiotics and reduce the number of genomic mutation sites. At the same time, it can increase the ecological stability of the intestinal microbiome and alleviate the fierce competition between probiotics and various species of Bacteroides in the local flora.

Understanding probiotic–host interactions is key to probiotics in the treatment of various diseases. These interactions include gut microbiota and immune system regulation, host gut barrier function, and increased production of gut microbiota-related metabolites such as SCFAs. Furthermore, probiotic genomics-especially multi-omics approaches-plays a pivotal role in comprehensively elucidating host–probiotic interactions and their respective mechanisms of action.[222] Han *et al.* found that *Bifidobacterium bifidum* M1-3 significantly improved DSS-induced colitis symptoms and alleviated intestinal barrier disruption in mice.[223] The fecal transplantation test confirmed that *Bifidobacterium bifidum* M1-3 is dependent on the gut microbiota for the relief of colitis. Multi-omics analysis revealed association between microbiota and metabolites, such as the significant increase in the level of 5-aminovaleraldehyde in the lysine degradation pathway after M1-3 treatment. In addition, gut microbiota has also been implicated in the pathogenesis of fatty liver disease, both in preclinical animal models as well as in patients. The transfer of gut microbiota to sterile animal models clearly indicates that the development of MALFD is determined by the gut microbiome, and there have been consistently observed in patients with fatty liver disease.[224] Jokisch *et al.* mentioned in their article that a partial liver resection experiment was performed on C57BL/6J mice fed a high-fat diet for 12 weeks, and 16S rRNA sequencing, metagenomics, metabolomics and other technologies were used to analyze the intestinal microbiota and liver metabolites, and it was found that the high-fat diet (HFD)-induced MAFLD mice underwent changes in intestinal microbiota and liver metabolism during liver regeneration, *A. muciniphila* It can reduce liver lipid accumulation and accelerate liver regeneration by regulating the tricarboxylic acid cycle.[225]

5. Biomedical applications and challenges

5.1. Biomedical applications in treating chronic diseases

5.1.1. Depression. Depression is a mood disorder that causes serious harm to physical and mental health, and its occurrence is closely associated with various biological abnormalities. Recent studies have demonstrated that the gut microbiota plays an important role in this process through interactions with the central nervous system *via* the microbiota-gut-brain axis.[226,227] Beaumont *et al.* were the first to propose the concept of the gut-brain axis, suggesting bidirectional signaling pathways between the gastrointestinal system and

the brain, and revealing the connection between emotional states and gut function.[228,229] Barandouzi *et al.* reported that the gut microbiota of depressed patients was significantly disrupted, and that targeted regulation of the microbiota could improve related symptoms.[230] Hsiao *et al.* demonstrated that oral administration of *Bacteroides fragilis* to the offspring of maternal immune activation (MIA) mice corrected intestinal permeability and microbial composition, while improving deficits in communicative, stereotypical, anxiety-like behaviors, and sensorimotor function.[231] Multiple probiotic preparations have been shown to alleviate depressive symptoms, and serve as clinical adjuvant therapies. A randomized controlled trial by Pinto-Sanchez *et al.* demonstrated that *Bifidobacterium longum* NCC3001 significantly improved depressive symptoms and quality of life in patients with irritable bowel syndrome, while also modulating brain responses in regions including the amygdala and limbic frontal lobes.[2] Disrupted tryptophan (Trp) metabolism is implicated as a key pathogenic factor in depression.[232] Studies have reported that transplantation of fecal microbiota from depressed patients into germ-free rats induced depression- and anxiety-like behaviors in the recipients and altered tryptophan metabolism.[233] The kynurenine (KYN) pathway is particularly crucial in this process. Rudzki *et al.* demonstrated that *Lactiplantibacillus plantarum* 299v reduced KYN concentrations in patients with major depressive disorder, thereby improving cognitive function and enhancing the efficacy of antidepressant medications.[25,234] Johnson *et al.* found that a probiotic combination containing *Bifidobacterium* W23 alleviated depressive-like behavior in high-fat diet model rats and decreased transcriptional levels of factors involved in the regulation of the HPA axis (CRH-R1, CRH-R2, and MR) in the hippocampus.[235] The regulation of monoamine neurotransmitters are also closely related to depression.[228,236] Erritzoe *et al.* discovered reduced 5-HT release in patients with major depressive syndrome, indicating impaired neurotransmission function.[237] The gut microbiota can influence 5-HT synthesis and release by regulating tryptophan metabolism. For example, *Bifidobacterium longum* E41 and *Bifidobacterium breve* M2CF22M7 can ameliorate chronic stress-induced microbial dysbiosis in mice, regulate TPH1 expression and 5-HTP secretion in RIN14B cells, and significantly reduce depressive-like behaviors in forced swimming, sucrose preference, and hypotension tests.[238]

5.1.2. Diabetes and obesity. Obesity is a condition with an increasing prevalence worldwide, often accompanied by multiple complications, especially type II diabetes (also known as non-insulin-dependent diabetes). This is because obese patients have excess adipose tissue and fat redistribution, which is directly related to hyperglycemia, hyperlipidemia, insulin resistance, endothelial dysfunction, and chronic inflammation.[239] Changes in gut microbiota are strongly associated with obesity, diabetes, and other chronic metabolic diseases. Studies have shown that dysbiosis, *i.e.*, an imbalance in the composition or metabolism of the gut microbiota, promotes inflammation, alters intestinal epithelial permeability, and produces signaling mediators that perturb fatty acid metabolism, thereby contributing to obesity.[240] Multiple clinical GWAS studies have shown that patients with T2D suffer from moderate intestinal dysbiosis, characterized by a reduction in butyrate-producing species of *Roseburia intestinalis* and *Faecalibacterium prausnitzii* and a relative enrichment of opportunistic pathogens.[241] Diabetes mellitus and obesity are both energy-regulating diseases, and the composition of the gut microbiota is able to regulate energy intake in the diet. Therefore, with proper probiotic and prebiotic treatment regimens, several issues of energy homeostasis can be addressed to alleviate the symptoms of diabetes and obesity.[242,243] Byrne *et al.* reported that dietary SCFA supplementation can increase the concentrations of circulating glucagon-like peptide-1 (GLP-1) and peptide YY (PYY).[244] John *et al.* showed that oral administration of live *Praxella* strains or extracts of *Prassobacterium* strains reduced fasting blood glucose levels and improved glucose tolerance in pre- and type 2 diabetic mice, compared to control mice.[3] Collectively, these findings suggest that probiotic administration is effective in preventing or ameliorating pre-existing prediabetes and T2D. In addition, Westfall *et al.* investigated and found that the combination of three probiotics (*Lactiplantibacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* NCIMB 5221, and *Bifidobacterium longum* subsp. infantile NCIMB 702255) with a novel polyphenol-rich prebiotic triphala had combinatorial benefits on the symptoms and underlying mechanisms of diet-induced diabetes and obesity.[242]

5.1.3. Inflammatory bowel disease. Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract (GIT), including ulcerative colitis (UC) and Crohn's disease (CD), in which patients tend to experience a decrease in gut microbiota diversity and stability.[245,246] IBD results in impaired intestinal epithelial barrier integrity, with a decrease in epithelial resistance and paracellular connexins such as claudin and occludin. During the exacerbation phase of IBD, the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α increases, and the TLR4 signaling pathway and toll-like receptors all affect the development of IBD.[247] When Bifidobacteria and Lactic acid bacteria were combined with Streptococcus or *Escherichia coli* Nissle 1917, relief of mild to moderate UC symptoms has been observed. Another study compared the treatment response of a mixture of Streptococcus thermophilus, four strains of *Lactobacillus* and three strains of *Bifidobacterium* (known as VSL#3) with placebo for UC and found that about 44.6% of participants treated with VSL#3 had a reduction in UC symptoms, compared with a 25.1% reduction in the placebo group.[248] Chen *et al.* functionalized halloysite clay nanotubes (HNTs) using the excellent adhesion activity and metal coordination ability of epigallocatechin gallate (MPN) and then incorporated them into alginate microspheres for probiotic loading.[249] MPN@HNTs targeting the inflamed colon site through electrostatic interactions, the rod-like microstructure of HNT prolongs its retention time on the mucosa and enhances its accumulation. In addition, MPN@HNTs not only reduce the aberrantly activated immune response by eliminating ROS, but also improves the probiotics' resistance to oxidative stress. Notably, oral administration of MHBSA can also modulate the gut microbiota, restore its

diversity and enhance the abundance of short-chain fatty acid-associated bacteria. Zou *et al.* developed a smartly engineered probiotic (IEP) capable of sensing biomarkers associated with enteritis disease and encapsulated them within polyserine-modified alginate (PA) gel microspheres, which were subsequently encapsulated with a hyaluronic acid (HA)-EGCG mucosal coating to form an EHmucus-encapsulated PA microsphere gel (MM) delivery system.[5] It effectively protects the harsh environment of engineered bacteria in the stomach, significantly improves intestinal adhesion of probiotics, prolongs colonization up to 24 hours, and does not affect the entry of biomarkers or the release of AvCystatin. Notably, its findings highlight the significant potential of IEPs sealed in the EH mucus-encapsulated PA Microsphere Gel Delivery System (IEP-MM) to provide continuous monitoring of IBD progression and mitigation phases through the bioluminescent activity of engineered bacteria in fecal samples. In addition, the IEP-MM system has demonstrated significant efficacy in the treatment of colitis by identifying disease biomarkers and regulating drug release accordingly.

5.1.4. Colon cancer. Colorectal cancer (CRC) is the third most common cancer worldwide, and its occurrence is inextricably linked to the gut microbiota. Increased abundance of a variety of gut bacteria, such as *Fusobacterium nucleatum* and *Streptococcus gallolyticus*, promotes the development of colorectal cancer. Probiotics can contribute to restore this balance, potentially reducing the risk of CRC. Recent studies have shown that probiotics and their specific metabolites can modulate the antitumor efficacy of chemotherapy and immunotherapy by shaping host immunity and balancing the gut microbiota. Engineered bacteria containing the cytolysin A protein (ClyA, a pore-forming cytotoxin) can act on tumor cells by forming transmembrane pores and inducing apoptosis.[250,251] An *et al.* isolated a novel bioactive protein, P8, derived from *Lacticaseibacillus rhamnosus*, which exhibits anti-CRC properties in both cell culture and xenografts.[252] In addition, He *et al.* found that gut microbial metabolites, especially butyrate, can directly regulate CD8 T cells through ID2-dependent mediation of the IL-12 signaling pathway to promote anti-tumor therapeutic effects.[253] Peng *et al.* showed that culture supernatant (CFCS) of *Lactobacillus dietaris*-free (LC-CLA) cells significantly reduced transcription levels of key genes for tumor cell growth and proliferation, such as CDK1/2/6, PLK1, and SKP2, and inhibited the viability of colorectal cancer cells (HCT-116).[254] In addition, daily consumption of LC-CLA for one week regulated the composition of the gut microbiota by specifically reducing the relative abundance of sulfur-producing bacteria in mice. Tumor-immune interactions play a key role in the treatment of colon cancer. T cells localize within the tumor epithelium to enable direct contact and are essential for antitumor function.[255] Yue *et al.* investigated the probiotic function of the *Lactiplantibacillus plantarum* YYC-3 strain and its cell-free supernatant (YYCS) during tumor development and growth in a colon cancer $APC^{Min/+}$ mouse model.[6] The results showed that YYC-3 inhibited colon cancer cells by regulating the immune system, downregulating the expression of inflammatory cytokines interleukin (IL)-6, IL-17F, and IL-22, and reducing the infiltration of inflammatory cells. In addition, YYC-3 was able to inhibit the activation of NF-κB and Wnt signaling pathways and restore altered gut microbiota composition. In addition, HDAC has become a new class of antitumor drugs by increasing histone acetylation in specific regions of chromatin, thereby regulating the expression and stability of apoptosis and differentiation-related proteins.[256] Li *et al.* used HDAC inhibitors to block the release of GRP78 secreted from colon cancer cells *via* exosomes by inducing its release from the endoplasmic reticulum.[257]

5.2. Challenges to industrialization

5.2.1. Technical bottlenecks of large-scale production. The design of current co-encapsulation systems has gradually expanded from traditional microcapsules to multifunctional composite carriers, but most research remains at the laboratory stage and is difficult to translate directly into industrially applicable products. This gap does not originate from the limitations of a single technology, but rather from systematic challenges faced by the entire manufacturing process across multiple stages including raw material preparation, process scale-up, online monitoring, and quality control.[66] Laboratory-scale production typically involves only a few grams to kilograms of raw materials, allowing for on-demand formulation, short mixing times, and precise control of conditions, such as temperature, humidity, and pH. In contrast, industrial-scale continuous production must handle tons of raw materials, which require advanced procurement and long-term storage. Mixing processes may last for several hours, and it is difficult to maintain laboratory-level precision over temperature and environmental conditions on production lines. These factors contribute to variability in the activity of each component in the final product.[258] For instance, metabolites such as vitamin B12 are prone to activity loss under high temperature, oxygen exposure, or extreme pH conditions.[259] Prebiotics (*e.g.*, fructo-oligosaccharides, inulin) are susceptible to moisture absorption and agglomeration during storage or enzymatic degradation during processing, which may alter their interaction patterns with probiotics.[1,260] Polyphenolic compounds readily form complexes with metal ions or emulsifiers, resulting in weakened antioxidant performance or migration losses.[261] Uneven concentration distribution of bioactive components during large-batch mixing may lead to locally excessive concentrations, compromising the viability or functional stability of probiotics.[262] Multi-component co-encapsulation also faces the challenge of increased difficulty in process parameter control, as changes in any single factor such as temperature, pH, or mixing intensity will simultaneously affect the stability of multiple components, necessitating consideration of the combined effects of multiple factors.[263] Quality control must expand from simple viable count and moisture content analysis to simultaneous monitoring of the concentration, activity, and interactions of all functional components. This requires integrated detection systems and real-time monitoring platforms. The combination of different functional components often requires adjustments in production processes, but most existing industrial equipment is designed for fixed products and

cannot easily accommodate the diverse formulation needs of co-encapsulation systems.

From an economic perspective, the industrialization of co-encapsulation systems also presents numerous challenges in cost control. The upfront research and development investment cycle is significantly extended, as multi-component optimization involves exponentially growing variable combinations, requiring extensive formulation screening, compatibility verification, stability evaluation, and efficacy confirmation experiments. Compared to single-component probiotic products, co-encapsulation systems typically have longer development cycles and incur substantially higher costs in labor, materials, and equipment. Furthermore, the increased regulatory certification costs exacerbate industrialization challenges, as multi-component products require safety evaluations for each functional component and their interactions, involving toxicological studies, functional verifications, and clinical trials that collectively exceed the requirements for single-component products. Different countries and regions have varying regulatory requirements for composite functional products, requiring companies to undergo multiple rounds of certification to meet global market access requirements.[264,265]

To address the bottlenecks in large-scale production mentioned above, future directions can be explored in the following areas: (1) adopting continuous manufacturing processes to replace traditional batch operations, which helps improve production efficiency and reduce batch-to-batch variability.[266] (2) Introducing process analytical technology (PAT) to provide real-time monitoring of critical quality attributes (*e.g.*, viable cell counts, moisture content, and functional component concentrations) for process optimization feedback, ensuring product consistency.[267] (3) Establishing a systematic quality control system covering raw material selection, strain cultivation, and final packaging to ensure products maintain long-term stability and safety during shelf life, while effectively preventing the introduction of allergens or other contaminants.[266] The implementation of these measures will help bridge the gap between laboratory research and industrial applications.

5.2.2. Standardization of safety evaluation. Conventional safety evaluation systems for probiotics primarily focus on individual strains and typically involve standardized testing procedures such as strain identification, antibiotic susceptibility testing, and virulence factor detection. However, co-encapsulation systems incorporate multiple bioactive components together with probiotics, making it difficult for traditional evaluation methods to comprehensively assess their safety risks. Functional components may modulate probiotic metabolic activities and intestinal colonization behavior, while probiotic metabolic processes can similarly affect the biotransformation, accumulation, and clearance of functional components. Additionally, when different strains are co-encapsulated, horizontal gene transfer, metabolic synergistic or antagonistic effects, and mutual interference during colonization may occur, further increasing uncertainties in safety assessment. Beyond interactions between biological components, the biocompatibility of encapsulation materials, their degradation products, and their interactions with probiotics must also be considered. Protective agents, stabilizers, and residual solvents used during encapsulation processes may also affect the safety of final products, particularly under conditions that pose risks of interference with probiotic viability and genetic stability. To address these challenges, various countries and regions have established their own regulatory standards. The U.S. Food and Drug Administration regulates probiotic safety primarily through the Generally Recognized As Safe (GRAS) system and the New Dietary Ingredient (NDI) framework, emphasizing historical evidence of safe use and scientific data. The European Food Safety Authority (EFSA) has implemented a stricter regulatory framework for novel foods and the Qualified Presumption of Safety (QPS) approach, which requires more comprehensive safety data. Japan's Ministry of Health, Labour and Welfare regulates such products through the Functional Food System, while China's National Health Commission relies on the Administrative Measures for New Food Ingredients. However, differences in classification criteria, assessment indicators, and approval pathways across these regulatory systems have led to the need for companies to repeatedly prepare documentation and conduct conformity verification when targeting different markets.[264,268] This situation increases the resource burden in product development and commercialization, and may lead to divergent safety conclusions for the same product due to inconsistencies in evaluation criteria.

Under current safety evaluation standards, special populations remain insufficiently addressed. Pregnant and lactating women are more sensitive to exogenous substances due to hormonal and metabolic changes.[269] Infants and young children face higher risks because their gut microbiota is not yet fully established, and their intestinal barrier and immune system are still developing. Although *Lactobacillus acidophilus* is generally considered safe in adults, cases of sepsis in infants suggest that this strain may cause serious adverse effects in this population.[270] In elderly individuals, impaired liver and kidney function, polypharmacy, and immunosenescence may reduce the clearance of probiotics, thereby increasing the likelihood of adverse reactions.[271] Immunocompromised individuals, such as organ transplant recipients, chemotherapy patients, and those with autoimmune diseases, may also struggle to effectively eliminate potentially harmful microbes due to impaired immune surveillance. These populations require stricter safety standards and prolonged monitoring.[268,272] Probiotics as functional foods or dietary supplements often need to be consumed over long periods to achieve their intended effects, but long-term use may alter gut microbiota composition, promote the horizontal transfer of antibiotic resistance genes, or induce chronic interactions with the host immune system, and these potential risks currently lack sufficient evidence to support definitive safety conclusions.[273] Therefore, establishing standardized safety evaluation systems tailored to co-encapsulation, improving risk assessment guidelines for special populations, and strengthening long-term safety monitoring are essential. At the same time, advanced approaches are being explored, such as *in silico* modeling to predict interactions between functional components and probiotics, and gut-on-a-chip systems that

more realistically mimic physiological conditions, thereby supporting stratified assessments of special populations and long-term safety studies.[274,275]

6. Conclusions and prospects

Co-encapsulation of probiotics with functional components (metabolites, prebiotics, and polyphenols) offers an effective strategy for improving probiotic delivery efficacy. The nutritional interactions, environmental modulation, and bioactive synergistic mechanisms between functional components and probiotics enhance the gastrointestinal survival rates, colonization efficiency, and therapeutic effects of encapsulation systems. The introduction of advanced manufacturing technologies including microfluidics, 3D printing, LbL encapsulation, electrospinning/electrospraying enables precise structural control and release behavior modulation of carrier systems. *Ex vivo* gastrointestinal simulation and *in vivo* imaging tracking provide analytical methods for functional evaluation, validating the application potential of co-encapsulation in chronic disease intervention. However, current studies remain largely confined to laboratory-scale investigations. Challenges persist in maintaining the stability of multi-component systems during scale-up, establishing standardized production protocols, and developing safety assessment frameworks tailored to specific populations. Future efforts should focus on the development of biocompatible materials, optimization of processing parameters, and refinement of clinical translation standards to advance the broader application of probiotic co-encapsulation systems in biomedicine.

Conflicts of interest

There are no conflicts to declare.

Data availability

Data availability does not apply to this article, as it is a review article and no datasets were generated or analyzed.

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