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Tapping the Potential of Probiotics: An Insight on Probiotic Delivery Systems, Their Quantification, and Assessment

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ABSTRACT

Background: Dysbiosis of the microbiota and gastrointestinal dysfunction are common characteristics of gastrointestinal diseases. Probiotics can alter the gut microbiota and serve as biological agents for treating digestive disorders. Although probiotics show promise in treating gastrointestinal disorders, several obstacles could hinder their effectiveness. These include concerns about safety, stress resistance, quantifying post-colonization, and evaluation methods.

Purpose: The aim of this review is to introduce the probiotic delivery methods and their mechanisms of action. It also described the functions of bacteriocins in providing probiotic strains with an advantage over competing treatments and the challenges associated with widespread deployment. It also evaluates current fluorescence-induced methods and numerous laboratory experiments involving living organisms' models for quantifying probiotics in complex microbiomes and assessing probiotic delivery systems.

Method: The approach includes a systematic analysis of recent developments in encapsulation technologies, delivery mechanisms, and innovative strategies for enhancing probiotic stability and functionality.

Result: The review found that encapsulation technologies have significantly evolved, with microencapsulation and nanoparticle systems being the most effective in ensuring the survival of probiotics through the gastrointestinal tract.

Conclusion: Probiotics could be used in gastrointestinal illnesses more effectively as therapeutic agents. This review has shown that effective delivery systems are critical to ensuring the viability and functionality of probiotics throughout their journey from production to gastrointestinal colonization. In conclusion, while substantial strides have been made in probiotic delivery, quantification, and assessment, continued interdisciplinary research and collaboration are essential to fully harness the benefits of probiotics in healthcare.

1. Introduction

Probiotics continue to face obstacles despite their considerable promise as therapeutic agents for treating gastrointestinal illnesses (Shen *et al.*, 2022). Probiotics must, first and foremost, be safe for human intake and free of transferable antibiotic-resistance genes (Sanders *et al.*, 2018). Therefore, it is typically not permitted to utilize modified probiotics for the treatment of diseases (Guevarra & Barraquio, 2015). Second, to be beneficial, viable counts must be at least 106 CFU/g. The most commonly utilized probiotics, however, are typically *Lactobacillus* and *Bifidobacterium*, which require better environmental conditions since they are more sensitive to aerophilous and high-temperature environments. Probiotics also have to tolerate bile and stomach acid throughout the gastrointestinal shift (Picard *et al.*, 2005). Probiotics may need to cling to the gastric outer layer and be possessed by the colon to be efficacious, even though they enter the colon alive. As a result, it might be difficult to create probiotic flora formulations for embattled distribution to the intestines. By encouraging the establishment of the productive strain in the gut, bacteriocins may function as signal peptides to gain an advantage over rival bacteria. Thirdly, there are still several issues with quantifying probiotics, including dynamic monitoring and on-site localization. The current feces-examining techniques fall short of the needs of rapidly expanding gut microbiota studies (Choudhary *et al.*, 2020). Novel techniques are therefore desperately needed, such as the ability to image

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gut bacteria and fluorescently mark them. Finally, more accurate *in vivo* and *in vitro* models must be developed to evaluate how well probiotic delivery systems work. The significance of probiotics delivery system lies in its critical role in advancing health and therapeutic interventions. Probiotics, beneficial microorganisms, are increasingly recognized for their ability to modulate gut health, boost immune function, and prevent various diseases. However, the efficacy of probiotics largely depends on their ability to survive in sufficient numbers through the gastrointestinal tract, necessitating the development of robust delivery systems. Effective quantification and assessment of these systems are vital for ensuring probiotic viability, safety, and therapeutic efficacy, thus enhancing their clinical and commercial application. This area of study bridges important gaps in the field of functional foods and personalized medicine.

2. Mechanism of Action of Probiotics

The therapeutic effects of probiotics might manifest through several different methods. Probiotics work by competing with commensal and pathogenic microorganisms, as well as by influencing immune responses and epithelial function. They can reduce the pH of the gut environment by increasing SCFA production, which prevents the growth of potentially dangerous microbes. Some probiotics improve the mucosal barrier's integrity, which normalizes intestinal penetrability (Gou *et al.*, 2022). The effects of probiotics range depending on their kind, dosage, and the various methods by which they interact with the host. Some produce chemicals like bacteriocins, hydroperoxides, lactic acid, and defensins, which have direct antibacterial effects. Others have non-immunological effects, such as denying nourishment to pathogens, producing more mucus, altering the pH of the intestines, encouraging the creation of tight junctions (TJs), or speeding up the healing process to lessen intestinal mucosal penetrability. Probiotics can impact the immune response by releasing fragments of their cell walls or DNA into the intestines (immunoglobulin synthesis, pro-inflammatory cytokine production). The synthesis and release of pro-inflammatory cytokines, such as interleukin (IL)-8, TNF-, and interferon-gamma (IFN-), are reduced, while the production of anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF), is enhanced. Moreover, they control the excessive activation of the nuclear factor kappa light chain enhancer of activated B cells (NFB) pathway (Markowiak *et al.*, 2017). Figure 1 depicts villus of the small intestine and probiotic modes of action.

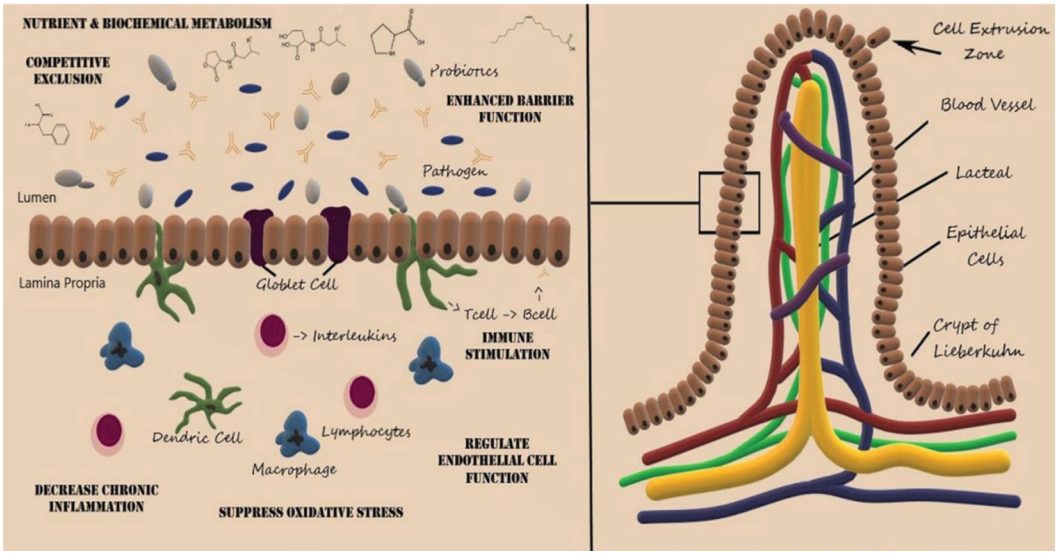

Figure 1: The villus of the small intestine and probiotic modes of action. Reprinted with permission under Creative Commons Attribution 4.0 International (CC BY 4.0).

3. Probiotics Function in Human Health: Biological Mechanisms

Through their biological actions in the body, probiotics have several positive effects on health. According to the "Human Microbiome Project," there are approximately ten times as many different microbial cubicles in the human body as there are human cells. These bacteria also include helpful microorganisms that are essential to maintaining human health. Probiotic bacteria yield postbiotics, including small-chain fatty acids, enzymes, and lactic acid. They also release antimicrobial peptides that can eliminate harmful

bacterial strains; however, the precise mechanism of action is not entirely understood. They are referred to as "natural preservatives" because of their bacteriocins (Juturu *et al.*, 2018). To counteract infections, hinder or prevent the attachment of harmful bacteria in the colon, and enhance the production of mucus, probiotics have also been linked to nutrients (Monteagudo-Mera *et al.*, 2019). Mucus creation, in turn, improves duodenal epithelial blockade, stimulating the immune response. Through additional enzymatic processes, probiotics boost the bioavailability of nutrients in the body and reduce pollutants through bile salt hydrolase (Allain *et al.*, 2018; Skrypnik *et al.*, 2019; Dubey & Patel, 2018). Even more, some probiotics may secrete unique anticarcinogenic and anti-oxidant metabolites that aid in the therapy of illness (Gulzar *et al.*, 2019). After receiving antibiotics, the gut flora can be replenished with the use of endogenous probiotic supplements. Probiotics can be used before, during, or after antibiotic therapy to prevent antibiotic-related diarrhoea (Rodgers *et al.*, 2013). Additionally, researchers have discovered that particular processes linked to probiotics' signalling of nerve activities in the central nervous system can support possible therapeutic effects on neuropsychiatric diseases brought on by stress (Sudo, 2019; Spencer & Hu, 2020). In addition to improving digestive health, probiotics have been shown to have effects on the brain (Bermúdez-Humarán *et al.*, 2019; Kim & Shin, 2019; Dalile *et al.*, 2019) treat irritable bowel syndrome; (Lavelle & Sokol, 2020) lower blood levels of low-density lipoprotein; (Nazir *et al.*, 2018; Yan *et al.*, 2019) prevent yeast and bacterial-related vaginal and urinary tract infections in women; (Akgül & Karakan, 2018; Atassi *et al.*, 2019; Van de Wijgert, 2019) avoid pancreatitis and recover pancreatic health; (Thomas & Jobin, 2019) improve respiratory tract strength; (Eguchi, 2019; Clua *et al.*, 2017; Percopo *et al.*, 2015) constrain tumorigenesis; (Konishi *et al.*, 2016) and control the immune system (Drago *et al.*, 2010). Probiotics also help with the treatment of cancer, (Galdeano *et al.*, 2019; Zhang *et al.*, 2019; Hendijani *et al.*, 2018) oral Candida infections, periodontitis, (Mishra *et al.*, 2019; Vasquez *et al.*, 2019) non-alcoholic fatty liver disease, and cardiovascular diseases (Aron-Wisnewsky *et al.*, 2020; Górska *et al.*, 2019; Shu *et al.*, 2020; Wong & Yu, 2019). The careful selection of the proper probiotic strains and genera is essential for various applications since different strains have unique health effects (Hu *et al.*, 2019; Nyvad & Takahashi, 2020). The metabolic processes of probiotics can be used to explain their biological impacts, which describe the implications of probiotics in the human body or their physiological effects. These outcomes are characterized as follows: Modulation Probiotics affect signalling pathways that either stimulate or decrease the control of processes, resulting in metabolic modulations (Aceti *et al.*, 2018). Intestinal epithelial cells' barrier activities can be modified by probiotics' binding capacity to the intestinal mucosa for cluster formation and their communication with distinctive immune responses, which benefits the host's health. (Sanders *et al.*, 2018; Plaza-diaz *et al.*, 2019). These modulatory mechanisms improve immunity (immune modulation) by acting as antagonists against pathogens. They also boost antioxidant potential, enhance intestinal transit, improve nervous system function (neuromodulation), control blood pressure and vascular endothelial Suez function, lower cholesterol levels, and sustain the active balance between healthy and injured cells (Bron *et al.*, 2017; Suez *et al.*, 2019; Michael *et al.*, 2017).

3.1. Synthesis

One of the most important functions of probiotics is the formation of active metabolites through their mode of action. For instance, the synthesis of vitamins (Vit-B2, Vit-K2, and Vit-B12 by B. subtilis and B. megaterium, respectively) by species-specific mechanisms and the synthesis of signalling molecules by strain-specific mechanisms are certain sorts of examples.

3.2. Absorption

Probiotics can improve the metabolism and bioavailability of micronutrients (Park *et al.*, 2018). Significant amounts of iron are absorbed in the form of Fe2+, but in the intestinal mucosa, iron interacts with apoferritin to change Fe2+ to Fe3+ (ferritin) (Vanhatalo *et al.*, 2018). Probiotics in the gut microbiota can aid in the conversion of Fe3+ to Fe2+, which increases iron absorption in the duodenum. According to Hoppe *et al.* (2015) extracellular enzymes increase the relative iron bioavailability and iron absorption rates when lactic acid bacteria are consumed. Similar findings were made by Dubey and Patel, who discovered that probiotics are significantly important for improving calcium uptake and absorption through intestinal fermentation. Probiotics play a role in the production and absorption of vitamin D, according to a different study by Costanzo *et al.* (2018). These illustrations clarify how probiotics work to increase the body's ability to absorb vitamins and minerals.

3.3. Prophylaxis

Probiotics' prophylactic mechanism aids in illness prevention and lowers the peril of contracting contagions, allergies, viral infections, and malignancies (Tungland, 2018; Gu *et al.*, 2016; Ballini *et al.*, 2019). The function of probiotic prophylaxis in the treatment of bacterial vaginosis has been described by Monteagudo Mera *et al.* (2019). Significantly, numerous interventional experimental trials are being carried out to determine the efficacy of probiotic prophylaxis.

Antimicrobial proteins and probiotic bacteria: Panigrahi *et al.* (2017) conducted randomized clinical studies to examine the efficacy of a symbiotic supplement in treating neonatal sepsis (Kumar *et al.*, 2020) noteworthy decreases in both positive and negative culture sepsis have been reported.

4. Targeted Probiotic Delivery

Probiotics delivered through dietary supplements, meals, and beverages have gained popularity as a result of people's constantly expanding health demands. Different populations with significant well-being maintenance needs, like newborns, children, teenagers, the elderly, and recovery from injury healing or surgery, are drawn to such dietary supplements and nutritionally enriched meals (Razavi *et al.*, 2021). Yogurt, ice cream, and dairy goods are among the functional foods with added probiotics that are most well-known, but the market for innovative non-dairy products has just lately begun to grow (González-Ferrero *et al.*, 2018). Additionally, unique consumables such as chocolate bars, cereal, drinks, and chips have been combined with desired strain combinations (Chen *et al.*, 2018).

Probiotics must be successfully incorporated into nutritional supplements or foods, which involves thorough testing to determine the best strain mix and delivery method. Before a flawless product can be produced for consumers, difficulties such as pH and aqua activity modification, temperature management, sensory concerns, and shelf-life evaluation must be resolved (Gani *et al.*, 2018; Kailasapathy *et al.*, 2014). Dairy products are excellent candidates for probiotic-enhanced diets because they often have low storage temperatures and large fat contents having favourable conditions for the incorporation of probiotics. Recently, probiotics have been stabilized in some matrices of foods or beverages using controlled and encapsulation release technologies, which are general among pharmacists (Anselmo *et al.*, 2016; Olnood *et al.*, 2015). As a result, there is now a greater variety of snacks that contain healthy probiotics. The targeted distribution of probiotics has gained popularity lately. The goal of such targeted delivery is often to get the probiotics into the colon to improve g.i.t., reduce lactose intolerance, relieve loose motions, promote protection against diseases, and lower fatty acids (Gheorghita *et al.*, 2021; Kim *et al.*, 2021; Li-Juan, 2013). It can be difficult to create probiotics that can withstand the harshly acidic gastrointestinal environment for targeted delivery to the intestines. Unprotected probiotics might suffer severe consequences from exposure to gastric acid, which is why it is necessary to use coating layers and encapsulating techniques to shield probiotics from a very low pH environment until they are transferred to their intended destination (Ta *et al.*, 2021). The most popular coating techniques include pH-sensitive and bacterium-sensitive encapsulated layers that could proclinate probiotics in the bowel to retort particular pH situations or particular bacteria clusters (Dodoo *et al.*, 2017). Utilizing usual and affordable coating materials, improving outside surface adhesion to intestinal epithelial cells, increasing the bioavailability, bile salt hydrolase activity, probiotic stability, antagonistic activity, effectiveness, targeting capacity of transport, and associated protective concerns are other crucial factors to take into account. The most often used wall components for achieving targeted administration of different probiotics include nutritional filaments, proteins, and natural polysaccharides (Singh *et al.*, 2018). Additionally, biocompatible artificial ingredients have been used. Emulsifiers are used as matchmakers when combining different wall materials to improve efficacy. More importantly, consideration must be given to the probiotic's stability, particularly during the drying stage of diet preparation (Devani *et al.*, 2017). Drying approaches used in this case often include electrospinning, extrusion, reflectance window drying, freeze drying, spray drying, and emulsifying. To select the best processing strategy, it's vital to consider the price and temperature of each drying procedure. In addition, cutting-edge methods, including microfluidics, 3D printing, and genetic engineering, have recently been used to improve encapsulation efficacy (Kevin *et al.*, 2021). Table 1 describes the recent probiotics delivery system.

Table 1: Recent Probiotics Delivery System

S No	Delivery system	Polymers	Probiotic stain	Functionality	References
1	Enteric coating spheres	Eudragit L100 55	*Lactobacillus casei*	Except for drying, there was no discernible decrease in viability (1 log loss of viable cells).	(De Barros *et al.*, 2015)
2	Bionanocomposites	PLGA, chitosan, and alginate	*Bacillus coagulans*	After microwave drying, B. coagulans had a high viability of 99.43%, and it degraded under stimulated gastrointestinal fluids at a rate of 94.76%. B. coagulans cells were protected by a nanocomposite made up of 57% BNC and 43% pectin from heat dry and GIT conditions. Nanocomposites added to bio nanocomposite formulation increased the steadiness of B. coagulans at various temperatures during long lasting storage.	(Asgari *et al.*, 2020)

3	Alginate capsule coated with chitosan	Chitosan	*Bifidobacterium breve*	Probiotic release over 240 minutes. Most viable cells recovered for a 3-layer coated matrix.	(Cook *et al.*, 2013)
4	Chitosan and CMC Polyelectrolyte complex	Chitosan, alginate	*Lactobacillus acidophilus*	Probiotic cells died under similar conditions, however, probiotic L. acidophilus encapsulated with 3 layers of CMC/ CHI/CHI showed a subsistence rate of roughly 33 % of cells. In the stomach environment, the multilayer structure demonstrated stability.	(Singh *et al.*, 2017)
5	Core-shell	Protamine, chitosan, alginate	*Lactobacillus casei*	The penetrating property of the shell was increased	(Garcia-Brand *et al.*, 2022)
6	Cell surface engineering	Carboxymethyl cellulose, chitosan	*Lactobacillus acidophilus*	The encapsulation efficiency was increased	(Priya *et al.*, 2011)
7	Microspheres	Chitosan-coated skim milk alginate	*L.rhamnosus* and *L.plantarum*	Encapsulation efficiency, tolerance, and storage stability were increased	(Padhmavathi *et al.*, 2023)
8	Macrogel	Starch & metal ions	*Lactobacillus paracei*	Strong digestive acid tolerance, exhibited extended controlled release possessions contrary to probiotics	(Sun *et al.*, 2023)
9	Hydrogel	Bentonite, alginate	*Lactobacillus rhamnosus*	The LGG survival rate at stomach pH values has increased. Following hydrogel breakdown, full intestinal LGG release was seen.	(Kim *et al.*, 2021)
10	Microcapsules	Polygamma-glutamic acid hydrogel	*Lactobacillus acidophilus*	The microcapsule maintains the intestinal mechanical barrier, distributes probiotics quickly, responds to NO, and controls the balance of the intestinal flora.	(Sun *et al.*, 2023; Altamirano-Ríos *et al.*, 2022)
11	Microgel	Methylacrylylated gelatin (GelMA), methylacrylylated hyaluronic acid (HAMA)	*Lactobacillus reuteri*	Defending against immune system assaults and reducing the risk of probiotics escaping are also important.	(Chi *et al.*, 2023)
12	Microbeads	Alginate Gum	*Lactobacillus rhamnosus*	Improve the survivability of probiotics for targeted delivery.	(Ali *et al.*, 2023)
15	Tablet	Bifidobacterium longum HA-135	*Lactoglobulin or Succinylated -lactoglobulin*	Incubated for 2 hours (acidic medium), succinylated lactoglobulin demonstrated survivals above 105 and 107 CFU. B. longum was not preserved in the acidic stomach juice by native lactoglobulin. These tablets exhibited stability after three months of refrigerator storage.	(Asgari *et al.*, 2020)
16	Tablet	Carboxymethyl strach with high amylose	*E. coli*	Compared to free probiotics or probiotics that are encapsulated in non-derivatized starch, there are higher viability rates in acidic stomach circumstances. after six months of refrigeration-stored storage, good viability	(Khorasani & Shojaosadati, 2017)
17	Microcapsules	Whey protein/ Pullulan	*Bifidobacterium animalis subsp. lactis*	On comparison with pullulan-based capsules, WPC-based capsules confirmed a higher level of cell viability. Bifidobacteria's lifetime was prolonged by electrospray encapsulation while being stored at 4 & 20 °C and at various relative humidity.	(Odila *et al.*, 2019)
18	Beads	Alginate, pectin, whey proteins	*B. bifidum*	Improved cell viability in capsules. After two hours (pH 2.5), probiotic cells did not survive, but there was only a two-log drop for the probiotics that were immobilized.	(Yeung, 2016)
19	Microspheres	Cellulose acetate Phosphate (CAP)	*Bifidobacterium pseudolongum*	In the simulated stomach environment, microencapsulated B. pseudopodium outlived non-encapsulated B. pseudopodium (109 cfu/mL).	(Liu *et al.*, 2020)
20	Tablets	Hydroxypropyl methylcellulose phthalate	*Lactobacillus fermentum*	Very less disintegration time and good cell feasibility were factors taken into consideration for tablet optimization.	(Villena *et al.*, 2015)

21	Nanocomposites	Bacterial nano cellulose, pectin, Schizophyllum commune	*Bacillus coagulans*	After microwave drying, B. coagulans had a high viability of 99.56 %, and it degraded under stimulated gastric fluids at a rate of 94.76%. B. coagulans cells were protected from heat drying and GI tract conditions by a nanocomposite containing 57% BNC and 43% pectin. The inclusion of BNC in the bio nanocomposite formulation also increased B. coagulans' stability at various temperatures during long-lasting storage.	(Khorasani & Shojaosadati, 2016)
22	Polyelectrolytes	Chitosan and CMC	*Lactobacillus acidophilus*	Probiotic cells died under similar conditions, however, probiotic L. acidophilus encapsulated with three layers of CMC/CHI/ CHI showed a subsistence rate of roughly 33 % of cells. In the stomach environment, the multilayer structure demonstrated stability.	(El-Sayed *et al.*, 2021)
23	Microcapsules	Chitosan coated alginate	*Lactobacillus acidophilus*	Yogurt with L. acidophilus-loaded CCAMs had better vitality than yogurt containing free cells suspended in it. Even probiotic cells that are naturally present in yogurt and not encapsulated probiotics can be more viable in SGF and SIF.	(Zanjani, 2014)
24	Microcapsules	Chitosan coated alginate	*Lactobacillus plantarum*	The encapsulated live probiotics were successfully kept alive over prolonged storage thanks to LP80-loaded GCCA microcapsules. Six months after encapsulation, the vitality of the probiotic cells was preserved. The encapsulated HepG2's metabolic activity demonstrated the potential of these GCAC microcapsules for cell treatment.	(Albadran *et al.*, 2015)
25	Microcapsules	Alginate, Xanthan, Carrageenan, Guar, or locust bean gum	*Lactobacillus rhamnosus, Bifidobacterium longum, L. etc.*	Under acidic conditions, coated bacteria showed a strikingly better subsistence rate than uncoated one. After being exposed to taurocholic acid, the feasibility of the free probiotic cells was decreased by 6.56 Log CFU/ml. Probiotic cells treated with alginate, xanthan gum, and/or carrageenan gum showed lower decreases of 3.23, 3.54, and 4.02 Log CFU/ml, respectively. Probiotics can be shielded from strong acidic conditions by xanthan gum, carrageenan, or alginate.	(Bevilacqua *et al.*, 2019)

5. Factors Affecting Probiotics Viability

Probiotics must remain viable to function, but doing so from the point of manufacture or delivery to the intended location in the gastrointestinal tract is challenging. Due to this, the mainstream of probiotic delivery systems studies concentrates on enhancing the probiotics' vitality. The factors that affect probiotic viability are covered in this section, including production, storage, and transit through the gastrointestinal tract.

5.1. Thermal Pressure

Probiotics' veracity can be harmed through heat strain that occurs both frequently and during long-term storage applicable manufacturing techniques, including pasteurization and drying (Hoppe *et al.*, 2015). Probiotics are well known to be negatively impacted by high temperatures *via* protein denaturation and ensuing cell inactivation damage (Costanzo *et al.*, 2018). The *Lactobacillus* species tested for heat tolerance for five minutes at 60°C, and their viability dropped by six log cycles, according to the results based on their sensitivity to heat.

5.2. Oxidative Stress

The feasibility of probiotics can be compromised by oxygen, since numerous probiotic strains are anaerobes or microaerophiles. Under oxidative conditions, reactive oxygen species (ROS) are produced and interact with probiotic components like proteins, lipids, and nucleic acids (Sadeghi-Bojd *et al.*, 2019). According to a study, the presence of oxygen hindered the growth of *Bifidobacterium* spp. In a different investigation, *Lactobacillus acidophilus* and *Bifidobacterium* species showed oxygen concentration-dependent toxicity (Chandel *et al.*, 2019; Wells & Mercenier, 2008).

5.3. Osmotic Shock

Probiotics are less viable when dried due to osmotic shock. Probiotic cells experience osmotic shock due to increased intracellular molarity as a result of dehydration that occurs during the drying process, which results in impaired cell activities (Panigrahi *et al.*, 2017). For instance, air drying and spray drying were found to have reduced the viability of *Lactobacillus plantarum*. With increasing hyperosmotic shock, Saccharomyces cerevisiae's vitality is reduced.

5.4. Gastric Juice

The hostile environment in the abdomen, more notably the highly acidic gastric fluid, remains the first and most significant barrier to preserving the survival of probiotics after ingestion. The pH of the stomach is typically between

1 and 2.5, and the gastric emptying period is roughly two hours. Due to disruptions in metabolic and cytoplasmic activity, probiotics cannot live in acidic environments for more than two hours. Since probiotics must travel through the stomach to reach their intended location, acid resistance is thought to be a necessary component of the efficient delivery of probiotics. pH sensitivity can be evaluated *in vitro* employing synthetic gastric fluid that has the same buffer capacity, osmolality, and surface tension as human stomach fluid.

6. Quantification of Probiotics using Fluorescence Labeling

Currently, high-throughput DNA sequencing of fecal microflora is heavily reliant on the identification and quantification of the majority of gastrointestinal microbiomes (Qin *et al*., 2010). The composition of the gut microbiota creates a steady environment where microbes work together and restrain one another. The ability of the fecal microbiome to fully capture the diversity of colonized bacteria in the gut is debatable (Donaldson *et al*., 2016; Tropini *et al*., 2017). However, there are still a lot of issues with probiotic quantification, like dynamic monitoring and on-site localization. Due to their inability to colonize the intestinal system, many foreign bacteria are expelled from the feces. More bacteria than the colonized gut microbiota is found in feces. The constantly evolving gut microbiota investigations cannot be satisfied by the current feces' examination techniques. To identify microbial populations and their existence in the digestive tract, new methods are therefore critically needed. Fluorescent tagging and imaging of gut microorganisms might be a good solution to this issue. The recent focus of research has been on the creation of practical fluorescent imaging techniques for bacteria. Bacterial gene transfer for fluorescent protein was first made (Lim *et al*., 2017; Whitaker *et al*., 2017). The majority of gut microbiota, however, is difficult to identify and culture in laboratory situations, which makes gene transfer difficult (Barbier *et al*., 2018). Fluorescence in situ hybridization (FISH) and other in-vitro quantifying methods based on microbial nucleic acid sequencing were consequently employed (Johansson & Hansson, 2012). The fact that FISH can only identify dead bacteria is problematic. Consequently, there have been numerous attempts to label microorganisms in vivo. Bacterial targeting probes are created by conjugating antibiotics with fluorescent dyes and can selectively identify bacteria in complicated samples because a few antibiotics can attach to the bacterial external membrane (Imai *et al*., 2019; Wang & Chen, 2018; Wang *et al*., 2017). A probe that is specific to gram-negative bacteria can be created using Cy3 dye and lipopolysaccharide-targeted polymyxin B. After being incubated with intestinal flora, the peptidoglycan-targeted vancomycin Cy3 conjugation inquiry could label Gram-positive bacteria. The toxicity of the probes is the main cause of concern. Drug resistance and host-microbiota disorders may result from bacteria being damaged by antibiotic-based imaging probes, even at low concentrations (Faber *et al*., 2016; Rivera-Chávez *et al*., 2016).

The metabolic labeling technique is therefore anticipated to address this issue. Bacteria could be identified during proliferation or energy production using metabolically-based mimic probes, such as artificial substrates or precursors. Additionally, it has been used to monitor the colonization and spatial distribution of the gut microbiota (Wang *et al*., 2019). Bacterial surface proteins could be used to build the ribonuclease A (RNase-A)-coated near-infrared sulfide quantum dots (PbS QD) (Chen *et al*., 2020). The fluorophore that may identify *E. faecalis, S. pneumoniae, E. coli, Klebsiella pneumoniae, Staphylococcus aureus, and P. aeruginosa* is linked to the filament temperature-sensitive protein (FtsZ) inhibitor oxazol-benzamide (Ferrer-González *et al*., 2019). However, this approach wouldn't work if the metabolic pathways of the bacteria were altered in the gut microbiota. Different metal-cation sterilizants are utilized because they have negative surface charges (Pasquina-Lemonche *et al*., 2020). For imaging bacteria, researchers also use contrast reagents tagged with metal cations. The near-infrared fluorescence cluster is joined with a zinc (II)-coordinated molecule, and the probe has a high specificity for Staphylococcus aureus-infected wounds (Leevy *et al*., 2008). The antimicrobial peptide G3KL can concentrate on the cell membrane and target gram-negative bacteria when combined with fluorescent dye (Gan *et al*., 2019). The methicillin-resistant Staphylococcus TBP-1 could target the cell membrane without causing drug resistance (Li *et al*., 2021). These probes are straightforward to produce and have simple chemical structures, but they share the same protective concerns as antibiotic-based probes. (Hu *et al*., 2020). In-vitro fluorescent imaging of bacteria is used with the positively charged dye MitoTracker Red (Maslov *et al*., 2018). However, the cost is high because a substantial volume is required for the intragastric delivery of in vivo labeling. However, some cation probes, such as cationic peptides, were able to quickly enter human cells and produce a lot of background noise (Bullok *et al*., 2006; Nekhotiaeva *et al*., 2004). In conclusion, the fluorescence tomography technique is extremely regarded in probiotic quantification, despite its drawbacks and weaknesses. It is frequently utilized in bacterial quantification and imaging due to its benefits of high sensitivity, accuracy rate determination, and low-slung charge. To address all the aforementioned issues, improved fluorescent investigations and labeling techniques are desperately desirable.

6.1. Models for Bacterial Growth Inhibition

Common pathogens that cause digestive system disorders include *E. coli, S. listeria, C. difficile, H. pylori, and C. albicans.* Many researchers have screened and assessed the possible efficiency of probiotics using conventional in-vitro measurements of the inhibition of probiotics on pathogenic bacteria. In a study, the effects of L. acidophilus on a strain of enteroaggregative E. coli (MDR-EAEC) demonstrated the potential antibacterial and antibiofilm activity of green synthesized silver nanoparticles against MDR-EAEC strains with antioxidant properties (Abishad *et al*., 2022). In another study, Ruiz *et al*. (2020) combined *Bifidobacterium longum* subsp. to test the antibacterial action against *E. coli, Cronobactersakazakii, Listeria monocytogenes, and C. difficile* in coculture trials. Researchers used infantis CECT7210 and oligosaccharides. They discovered that the novel symbiosis may be a useful complement to infant health (Ruiz *et al*., 2020). By using the agar diffusion assay and broth inhibition assay methodologies, Cizeikiene & Jagelaviciute (2021) assessed the antibacterial properties of 12 pathogenic strains from the S. aureus, E. coli, S. chromogenes, and S. hyicus species. The findings supported the probiotic candidate's L. acidophilus DSM 20079, Bifidobacterium pseudopodium DSM 20099, and Bifidobacterium animalis DSM 20105 (Cizeikiene & Jagelaviciute, 2021). Pathogenic genes or genes linked to host infection were also used for probiotic screening in addition to spotting pathogen growth. Bifidobacterium longum JDM301, according to Wei *et al*. (2018) not only had a significant impact on the growth inhibition of *Clostridium,* in addition, D. difficile directly promoted the breakdown of clostridial toxin (Wei *et al*., 2018). By measuring the levels of important lipid metabolism genes, cytokines associated with inflammation, and biomarkers, Ghadimi *et al*. (2021) assessed the probiotic effects of Bifidobacterium and Malis R101-8 and showed that B. Through the molecular and signaling pathways set off by pro-inflammatory bacteria and lipids, R101-8 can enhance indicators of meta-inflammation (Ghadimi *et al*., 2021). Additionally, various silicon models have been created for the evaluation of probiotic function due to the swift progression of large-scale data science and bioinformatics. Metabolic models at the genome size and mathematical models have been used to predict and assess probiotic bacterial functions (Arciero *et al*., 2010; Choi *et al*., 2020).

6.2. Models for Simulating the Intestinal Microbiota In Vitro

Probiotic-pathogen interactions may be studied using a more contemporary batch fermentation technique that simulates the distal colon. For the simulation of the human intestinal microbiota, models including SIMGI, TIM-2, SHIME, ECSIM, PolyFermS, and EnteroMix have been developed (Gościniak *et al*., 2022). Although the majority of these models were developed to examine the relationships between food efficiency variables or medications and the abdominal microbiota, they have also been used to examine how probiotics, prebiotics, and symbiotics affect the gut microbiota. By employing SHIME, it was discovered that probiotic, prebiotic, and symbiotic therapies had a favorable modulating effect on the gut microbiota and metabolic activity of kids with autism spectrum disorder (Duque *et al*., 2021). MegaSporeBioticTM, an oral spore-based probiotic supplement containing five different species of Bacillus, was studied by Marzorati *et al*. (2021) for its effects on Using the SHIME, they assessed the activity of the gut microbiota and the composition of the community. They discovered that during treatment, Lactobacillus spp. decreased and Akkerman-Sia muciniphila, Bifidobacteria spp., and Firmicutes increased. Bacteroidetes also declined (Marzorati *et al*., 2021). The usefulness and viability of in-vitro gut microbiota simulation models are essential for further examination due to the paucity of studies.

6.3. Organoid and Cell Models

It is possible to research the interactions between probiotics and pathogens in the gastrointestinal tract using a variety of models that replicate the human intestinal epithelium. Culture plates and Transwell inserts are common 2D models, while more recent 3D models, including organoids, enteroids, and organ-on-a-chip, have been developed to evaluate probiotic-pathogen interactions (Anjum *et al*., 2022). Chen and colleagues discovered that H. Using an in vitro cell-based model, treatment with the Lactobacillus strains significantly reduced pylori adherence, the invasion of gastric epithelial cells, and interleukin-8 production (Chen *et al*., 2019). Bifidobacterium longum JDM301 was shown by Wei *et al*. (2018) to mitigate some of the tissue harm brought on by C. difficile while reducing the amount of C. employing in-vitro cell models to measure levels of toxins and C. difficile. In an organoid-dendritic coculture, Engevik *et al*. (2021) evaluated the function of Lactobacillus reuteri in influencing the host immune system and showed that both L. The development of dendritic cells can be aided by Reuters-released substances and their bacterial counterparts (Engevik *et al*., 2021). Ex vivo models have also been created, including the Human Intestinal In Vitro Organ Culture (IVOC) Model, the Ussing Chamber, and the Microbiota-human Intestine on Chip (MihI-oC) Model (De Gregorio *et al*., 2022). A "body-on-a-chip" is being developed by working to create larger systems that connect several organotypic models (Marx *et al*., 2021).

6.4. Animated Models

Animal models offer extremely measured conditions and permit the use of sterile animals for research on possible diseases as well as host-microbe interactions. Additionally, animal models offer the chance to gather samples from various GI tract regions, which is not conceivable during scientific studies. Though rats and mice are the most popular models, probiotic-pathogen interactions have also been studied in Caenorhabditis elegans, honey bees, Ciona robusta, fruit flies, and larger wax moths. According to Chen *et al*. (2020) colonization of H. lactobacillus strains has been shown to reduce pylori and cause stomach inflammation. G. was utilized by Scalfaro *et al*. (2017) (Mellonella) to assess L.'s capacity to fight germs. These findings revealed that G. rhamnosus GG and Clostridium butyricum Miyairi were effective against three intestinal bacteria that cause illnesses. A promising in vivo model that can support in vitro experiments to screen putative probiotics is mellonella larvae (Scalfaro *et al*., 2017).There are still few instances of genome-edited animals being employed in probiotic-related research, even though genome editing technology has advanced quickly and there are numerous genome-edited rats and mice in existence. Therefore, more research using animal models is required. There are three ways to check and evaluate the probiotics' potential effectiveness. Probiotics' ability to suppress the growth of harmful bacteria in vitro using pathogenic genes or host infection-related genes Models at the mathematical and physiological scales of clinical trials involving humans. Even though in-vitro models and animal experiments have the benefits of ease of use, controllable experimental conditions, and inexpensive research costs, reliable proof of the influence of probiotics on human health still heavily depends on clinical trials involving humans. Through human clinical trials, numerous studies have also helped to advance the development and commercialization of probiotic goods. For instance, E. There are numerous strains of E. coli, the majority of which are categorized as opportunistic pathogens. Escherichia coli Nissle 1917 is widely utilized and approved as a probiotic, which is mostly due to human clinical research (Zhao *et al*., 2022). In their comprehensive study of probiotic clinical studies, Dronkers *et al*. (2020) discovered that *L. Bifidobacterium animalis* ssp. and *Bifidobacterium rhamnosus* GG. The most extensively researched probiotic strain is Lactis BB12 (Dronkers *et al*., 2020). The benefits of probiotics for gastrointestinal illnesses have been the subject of numerous clinical trials, but the results have been uneven and occasionally contradictory. This may be due to a variety of factors, such as trial design, cluster size, bulk characteristics, and dose. Therefore, probiotics, the mass population, and study projects should all be sensibly taken into account when designing human clinical studies.

7. Advancement in Preclinical and Clinical Studies on Probiotics

Advancements in preclinical and clinical studies on probiotics have illuminated their potential therapeutic benefits across a range of health conditions. Preclinical studies have demonstrated the ability of probiotics to modulate the gut microbiota, enhance intestinal barrier function, and regulate immune responses, offering insights into their role in diseases such as inflammatory bowel disease (IBD), metabolic syndrome, and even certain cancers. Animal models have provided evidence that specific probiotic strains can attenuate inflammation by influencing cytokine profiles, reducing oxidative stress, and modulating gut-brain interactions, which are pivotal in neurodegenerative disorders like Parkinson's disease. Clinically, probiotics have been investigated through randomized controlled trials (RCTs) for their efficacy in treating conditions like irritable bowel syndrome (IBS), antibiotic-associated diarrhoea (AAD), and metabolic disorders such as obesity and type 2 diabetes. Strains like *Lactobacillus rhamnosus* GG and *Bifidobacterium* species have shown particular promise in reducing IBS symptoms and preventing AAD. However, variations in study design, probiotic strains, and dosages complicate the interpretation of results, highlighting the need for standardized clinical trials to optimize therapeutic use.

8. Recent Patents and Formulation of Probiotics

Recent patents on probiotics reflect significant developments in their therapeutic applications and delivery systems. Innovations have focused on improving the stability and efficacy of probiotic formulations, such as microencapsulation techniques that enhance bioavailability in functional foods. Target specific *Bifidobacterium* and *Lactobacillus* strains for gut health, particularly in conditions like irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). Probiotic patent with patent no. EP 3,381,601 explores the modulation of the immune system through probiotics, with potential applications in neurological and metabolic disorders. These patents underscore the growing role of probiotics in addressing diverse health conditions through novel strain selection and improved formulation methods. A list of patents has been described in Table 2.

Recent formulations of probiotics focus on enhancing stability, efficacy, and targeted delivery. One such example is microencapsulation technology which protects probiotics from stomach acid, improving their viability in functional foods and supplements. Another innovative formulation includes symbiotic combinations,

where probiotics are paired with prebiotics to enhance their growth and effectiveness, targeting oral and dental health. Additionally, time-release capsules, ensure a controlled release of probiotics into specific areas of the gut, optimizing their therapeutic benefits for conditions like inflammatory bowel disease (IBD).

Table 2: The list of some recent patents on probiotics, and a brief description of their innovations

Patent number	Title	Description	Year	Reference
US 10,857,104	Probiotic Compositions for Treating Gut Health	Covers specific *Bifidobacterium* and *Lactobacillus* strains for improving gut health, especially in IBD and IBS.	2020	(USPTO, 2020)
EP 3,381,601	Probiotic Composition for Immune System Modulation	Describes a probiotic formulation that enhances immune response by modulating gut-brain axis interactions.	2021	(EPO, 2021)
US 10,166,198	Microencapsulation of Probiotics	Focuses on a novel microencapsulation technique to improve probiotic stability and bioavailability in foods.	2019	(USPTO, 2019a)
US 11,103,514	Probiotic for Treatment of Metabolic Syndrome	Involves specific probiotic strains for reducing risk factors associated with metabolic syndrome and obesity.	2021	(USPTO, 2021)
WO 2020/045638	Probiotic Formulations for Oral and Dental Health	Probiotic strains aimed at improving oral and dental health by reducing pathogenic bacteria in the mouth.	2020	(WIPO, 2020)
CN 110321924 B	Probiotic Strain for Mental Health Support	Discloses a strain that targets the gut-brain axis to alleviate symptoms of depression and anxiety.	2021	(CNIPA, 2020)
US 11,282,921	Probiotic Use in Neurological Disorders	Focuses on the use of probiotics to treat or mitigate neurodegenerative diseases by modulating gut bacteria.	2022	(USPTO, 2022)
US 10,485,921	Probiotics for Reducing Antibiotic-Associated Side Effects	Describes probiotic compositions for preventing or mitigating antibiotic-associated diarrhea (AAD).	2019	(USPTO, 2019b)

9. Conclusion

Even though probiotics can be used to treat gastrointestinal disorders, there are still several obstacles that could prevent their widespread use. Probiotics will be used in gastrointestinal illnesses more effectively if the problems that are currently present are understood. Importantly, if probiotics cannot reach the intended location, the desired effect will not occur. Probiotics that produce bacteriocins can help preserve gut health and microecological balance. Still, problems with their large-scale production and instability in some settings prevent them from being used more widely. Fluorescence imaging technology has been a worry for probiotic quantification; to address the current issues, improved techniques for fluorescent probes and labelling are needed. So far, numerous models have been conducted within and outside of living organisms, including those that limit bacterial growth, simulate the gut microbiota in vitro, use cell and organoid models, animal models, and human clinical experiments to evaluate the potential efficacy of probiotics. These replicas are still in the early stages of development; thus, better and more accurate models must be created.

Abbreviations

Abbreviation	Full Form
IBD	Inflammatory Bowel Disease
RCT	Randomized controlled trial
AAD	Antibiotic-Associated Diarrhoea
IL	Interleukin
TGF	Transforming Growth Factor

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Ms. Anupama Choudhary envisaged the idea, carried out literature review, and prepared initial draft of the manuscript. Mr. Prince Rohilla and Mr. Gaurav Rohilla supported in carrying out literature search and compilation of data.

Dr. Minkal Tuteja executed language corrections and enhanced technical aspects of the manuscript, and refined the final version of manuscript.

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There are no conflicts of interest with this work, according to the authors.

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