

Colon Targeted Delivery Dosage Forms for Probiotics: A Review

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ABSTRACT

Encapsulation was a promising method for protecting probiotics from extreme conditions during their passage through the gastrointestinal tract and delivering probiotics to specific sites in the colon for colonization. Various dosage forms have been used in recent years to encapsulate probiotics to maintain cell viability during processing, storage, and through the digestive tract to provide health benefits. However, research related to the encapsulation of probiotics as the dosage forms for colon-targeted delivery systems was still quite limited to conventional dosage forms due to the sensitivity of probiotics to extreme conditions during the process. This review focuses on various types of dosage forms that are used in colon-targeted delivery systems for commonly used probiotic bacteria. In this review, we discussed the limitations of the current dosage forms used in probiotic encapsulation, along with the latest advancements in colon-targeted delivery systems for probiotic products. This review also covers future perspectives on the potential dosage forms that can effectively maintain probiotic viability and provide specific release in the colon.

Keywords: colon targeting delivery; probiotics; dosage forms; encapsulation

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INTRODUCTION

Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to humans by improving the balance of the intestinal microflora, restraining the growth of pathogens, including stimulation and maintenance of the immune system (Qin et al., 2021; Zheng et al., 2017). Probiotics are one of the supplements that are commonly used to treat diarrhea (Hill et al., 2014). The role of probiotics in maintaining the balance of intestinal flora during diarrhea is associated with various mechanisms, such as competing for adhesion sites and nutrients that inhibit the growth of pathogens. Probiotics have been observed to induce an increase in intestinal mucus secretion, thus enhancing the barrier function of the mucosa by promoting the function of tight junctions in the intestinal epithelial cells. (Plaza-Diaz et al., 2019). Increased mucin secretion from intestinal epithelial cells inhibits pro-inflammatory cytokine expression and promotes protective cytokine expression (Yang et al., 2020). Many types of bacteria can be utilized as probiotics, especially from the family of *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Bifidobacterium*, and *Bacillus*, which were found as the normal flora in the human intestine (Anselmo et al., 2016; Li et al., 2018; Paula et al., 2019). Probiotics need cell viability of at least 10⁷ CFU to show health benefits after passing through the gastrointestinal tract and colonizing the distal ileum and colon (E Silva et al., 2013; Sun & Wicker, 2021). This is

indicated by the number of bacterial cells in each dose of probiotic products on the market, typically ranging from 10⁷ to 10⁹ (Asgari et al., 2020).

Unfortunately, most probiotics are not resistant to conditions that occur during processing, distribution, storage, and during their passage through the gastrointestinal tract (pH of stomach acid, bile salts, and enzymes in the small intestine) (Torp et al., 2022). About 60% of ingested probiotics die before reaching the large intestine due to harsh environments, such as acidic stomach pH and the presence of bile salts (Singh et al., 2021). To enhance the effectiveness of probiotics in challenging environments, various strategies have been implemented. These include selecting bacterial strains that can withstand acidic conditions and bile salts, using appropriate packaging methods, stress adaptation, adding micronutrients, and encapsulating probiotics (Razavi et al., 2021).

Probiotic encapsulation is one of the promising solutions by retaining probiotics in the encapsulation matrix, which acts as a physical barrier protecting probiotics from adverse environmental conditions and delivers them to where they are wanted (Raisz et al., 2020). Several studies have demonstrated that encapsulation can improve the probiotics' viability in the digestive tract. It was found that encapsulating *Lactiplantibacillus plantarum* in calcium alginate capsules increased cell viability from 18.50% to 84.50% through *in vitro* tests

(Surono et al., 2018). In order to improve probiotic viability while passing through the gastrointestinal tract, a targeted delivery system to the colon can be used to provide a specific release of probiotics in the large intestine (Kumar et al., 2020). Various mechanisms exist for colon-targeted delivery systems, including time-dependent or pH-dependent systems, nanoparticulate systems, and utilizing the enzyme activity from colon microflora (Iswandana et al., 2021).

Lately, researchers have made significant progress in developing dosage forms that contain probiotic products, with a focus on optimizing colon-targeted delivery systems. These systems are designed to withstand extreme gastrointestinal conditions and ensure the specific release of probiotics in the colon, which aids in their colonization. Particulate or multi-particulate systems such as pellets, microspheres, beads, and nanoparticles have been used to formulate colon-targeted drug delivery systems (Iswandana et al., 2022). These systems are utilized based on their advantages; for example, pellets and beads provide a controlled release profile and flexibility in dosing, while a sustained release in colitis sites with a thick layer of mucus can be provided by microparticles. Besides that, nanoparticles can be accumulated for a long time in the ulcerated area of the colon (Iswandana et al., 2018, 2022).

Since the physiological condition of the gastrointestinal tract varies in every individual, most studies combine several systems to generate optimal colonic delivery of drugs (Iswandana et al., 2022). Unfortunately, there have been no reviews regarding the advancement of dosage forms used in the colon-targeted delivery of probiotics. Therefore, this paper will summarize an updated overview of current research regarding different colon-targeted dosage forms to deliver probiotics. To better understand the mechanisms of action of various dosage forms, it is necessary to review the appropriate methods for formulating these forms, as well as the physiological conditions of the gastrointestinal tract. In addition, the current limitations and future development trends of probiotic dosage forms are also discussed. We hope this review will bring a better understanding regarding the recent advancements in colon-targeted delivery systems for probiotics and encourage the pharmaceutical industries to explore suitable dosage forms to optimize the delivery of probiotics.

RESULTS AND DISCUSSION

Factors Affecting Probiotic Delivery by Oral Route

The oral route is the most used method for the administration of drugs, which might be intended to be absorbed into the systemic circulation or for local therapy in the gastrointestinal tract (Kurakula et al., 2021). The

absorption of orally administered drugs can be influenced by several factors such as the physicochemical properties of the drug (like pH-pKa value, partition coefficient, intrinsic solubility, polymorphism, etc.) and biological factors (like pH conditions of the gastrointestinal tract, presence of enzymes, amount of blood flow at the absorption site, absorptive surface area, presence of mucus, intestinal microbiota, etc.). Different anatomical or physiological conditions in each part of the digestive tract will affect the absorption and the drug dissolution rate (Hua, 2020).

The digestive system is a complex network of specialized organs that work together to break down food and absorb nutrients, electrolytes, and water. It is estimated that about 10 liters of material pass through the digestive tract each day. However, only 1.5 liters of fluid actually reaches the colon, and just 100 mL is excreted with feces (Kiela & Ghishan, 2016). The residence time and pH changes along the digestive tract became two main factors to be considered in designing a colon-targeted drug delivery system (Arévalo-Pérez et al., 2020). Figure 1 illustrates the physiology of the organs in the digestive tract and various factors that affect drug delivery via the oral route.

Dosage Forms for The Targeted Delivery of Probiotics to Colon

Consuming a daily intake of 10^7 - 10^9 CFU/g is necessary to achieve the desired probiotic benefits, which requires a minimum probiotic amount of 10^6 - 10^7 CFU/g (E Silva et al., 2013; Sun & Wicker, 2021). Recently, the interest in developing pharmaceutical preparations like capsules, granules, tablets, pellets, and microparticles containing probiotics has increased. Unfortunately, most probiotic products have a short shelf-life, even when stored at low temperatures. To overcome this problem, encapsulation technology comes as a promising solution to transform probiotic cells to be ideal to consume (Terpou et al., 2019). Encapsulating live probiotics in matrix systems can provide enhanced protection against harsh environmental conditions during transport, storage, and gastrointestinal tract passage. Various encapsulated dosage forms have been developed to promote the viability of probiotic cells and facilitate their delivery to the target sites, to confer the desired effect on health.

1. Microparticles

Microparticles with particle sizes ranging from 1 to 1000 μm , generally formulated in the forms of microgels, microparticles, microbeads, microspheres, and microcapsules, have been studied for colon-targeted purposes. This dosage form comes with the benefits of providing protection for probiotics from cell death and achieving a sustained or controlled release profile (Nidhi et al., 2016). Microparticles have been extensively explored and show promising protection of probiotics

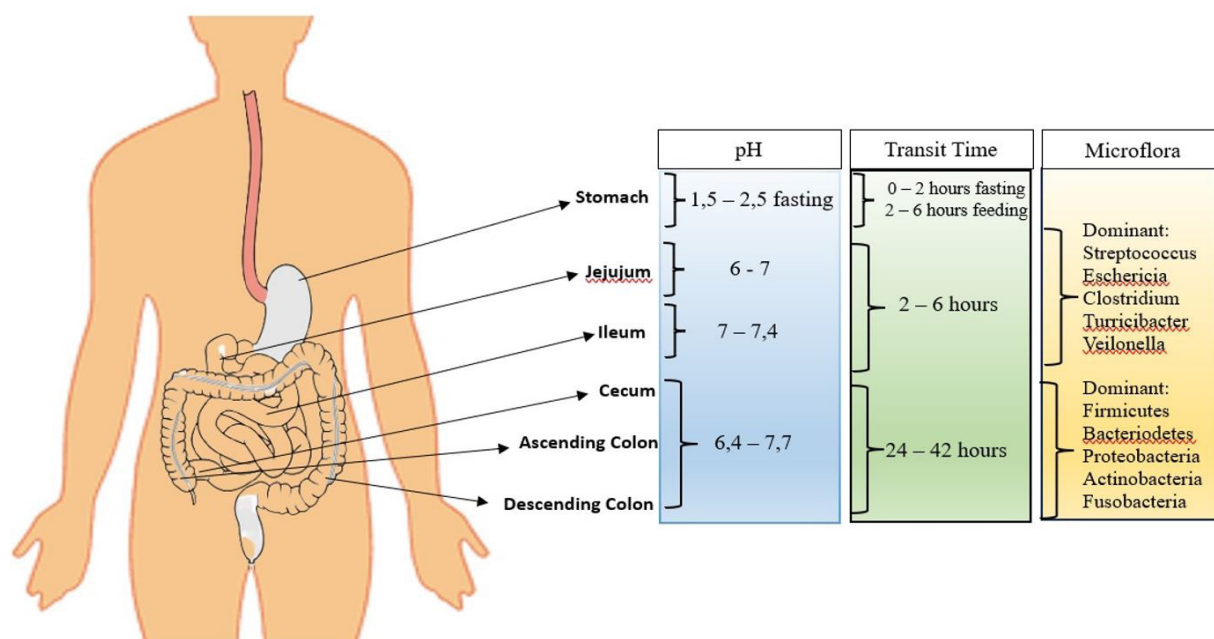


Figure 1. Physiology of gastrointestinal tract (Kurakula et al., 2021 with modification)

from extreme environmental conditions as well as maintaining their viability. Recently, encapsulation of *Bifidobacterium longum* using emulsion and extrusion techniques has demonstrated efficient protection of probiotic cells when further evaluated against heat exposure and sequentially exposed to simulated gastric fluid and intestinal fluids, as well as the low temperature while stored in a refrigerator.

In another study, the agent used to encapsulate probiotics was *Eleutherine americana* because the corresponding oligosaccharide extract of *E. americana* is resistant to α -amylase enzymes and low pH conditions. In addition, encapsulating probiotics using the extrusion method provides a better survival rate of probiotic cells in extreme conditions than encapsulating using emulsion technology. During the heat exposure test at 65°C for 15 minutes, probiotic encapsulation resulted in better cell viability than the free cells. This study showed that microencapsulation of probiotic cells using *E. americana* as an encapsulating agent provides better stability to their viable cells and could target the delivery of probiotics to the colon (Phoem et al., 2015). Other examples of microparticle dosage forms used for colonic encapsulation and targeting of probiotic cells using proteins and polysaccharides are shown in Table 1.

The electrospray method has been widely used as a promising method for encapsulating probiotics (Gomez-Mascaraque et al., 2016; Librán et al., 2017; Zaeim et al., 2017). Other encapsulation methods, such as emulsion, extrusion, and spray drying, need more attention while being used because they often reduce cell viability due to

high temperatures or cross-linked polymers. Meanwhile, the electrospraying method does not use excessive heat, which is safe for cell viability. Core-shell microcapsules can also be formulated using the electrospray method to provide optimal protection for the encapsulated probiotics. A study showed that the encapsulation of probiotics into core-shell microcapsules using alginate-zinc can increase the number of surviving probiotic cells in gastric fluid up to 5 times more than free cells (Laelorspoen et al., 2014). This technique, which requires only one or two steps of formulation, is relatively easy in formulating probiotics to the microparticulate dosage forms (Zaeim et al., 2017). Therefore, the dosage form effectively maintained its viability during transit in the gastric and intestinal tracts, thus providing targeted delivery of probiotics to the colon.

2. Pellets

Pellets are multi-unit pharmaceutical dosage forms characterized by spherical or pseudo-spherical shapes with smooth surfaces, and sized generally around 500 to 1500 μm , with relatively high density, and excellent flowability (Bipin & Jagdish, 2017). There are various methods for producing pellets, including wet extrusion (also known as extrusion/spheronization), direct pelletization (using a high-shear mixer or fluid bed), or hot melt extrusion. Additionally, other techniques, such as coating the drug powder/suspension/solution onto an inert core (powder, suspension, and layering solution), can be used. Compacting the powder into small tablets, known as mini tablets, can also be another alternative method (Palugan et al., 2015).

Table 1. Various dosage forms have been developed for the colon-targeted delivery of probiotics

| Main Encapsulating Excipient | Probiotic | Dosage forms | Result | Reference |
|------------------------------|---|---------------|---|--------------------------|
| Shellac & Alginate | <i>Limosilactobacillus reuteri</i> TMW 1656 | Microcapsule | The viability of probiotic cells was improved by encapsulating them in shellac and alginate microcapsules, which maintained their viability during freeze drying, heat exposure, storage, and simulated digestion. The addition of whey protein isolate (WPI) had a synergistic effect. | (Huang et al., 2021) |
| Alginate & whey protein | <i>L. casei</i> | Microparticle | The viability of microparticles containing probiotics was 6.20 – 9.77 log CFU/g after 3 hours of incubation in simulated gastric fluid, compared to 2.68 log CFU/g for the free cells. | (Smilkov et al., 2014) |
| Pectin & Starch | <i>L. plantarum</i> | Microsphere | The viability of encapsulated probiotics after incubation in simulated gastric fluid and bile salt solution was higher than the free cells. | (Dafe et al., 2017) |
| Alginate & Chitosan | <i>L. casei</i> | Microparticle | The microencapsulated <i>L. casei</i> viable probiotic cells were found to be more preserved and have a higher targeted release effectiveness. | (Ivanovska et al., 2017) |
| Alginate & silica | <i>L. rhamnosus GG</i> | Microparticle | Alginate-silica microparticles protect probiotics from low-pH stomach acid and promote better release and colonization in the colon. This delivery system is more effective than free cells. | (Haffner et al., 2017) |
| Pectin & CAP | <i>Saccharomyces boulardii</i> | Pellet | The CAP-coated pellet formulation has promising potential in enhancing the viability of probiotics, providing targeted drug delivery of mesalamine and <i>S. boulardii</i> to the colon as well as increasing the effectiveness of mesalamine in the management of ulcerative colitis. | (Singh et al., 2021) |
| Shellac | <i>Bifidobacterium bifidum</i> INT B2 | Granules | Encapsulated probiotics degraded less than 5% for 24 hours at pH 1.2 and 6.8. Whereas at pH 7.4, the loaded probiotics provide complete degradation after 10-11 hours. In the stability study, the viability of the granules decreased to 57.8% after five months of storage. | (Gately & Kennedy, 2017) |
| Soy protein, alginate & HPMC | <i>L. casei</i> | Tablet | Tablets containing probiotics maintained their minimum viability above 10 ⁷ CFU/g during incubation in simulated gastrointestinal fluids for 4 hours, providing slow release in the intestine. Tablet viability after storage stability test at 25°C/60% humidity for 42 days was 10 ⁶ CFU/g. | (Hadzieva et al., 2019) |

Table 1. continued

| Main Encapsulating Excipient | Probiotic | Dosage forms | Result | Reference |
|---|--|----------------|--|--------------------------|
| Polymethacrylate Eudagrit RS 100 | <i>Lactobacillus acidophilus</i> | Microparticles | Enteric microparticles were resistant to simulated gastric fluid conditions and could release probiotics contained in simulated intestinal fluids during <i>in vitro</i> tests. The enteric microparticles were also stable in the long-term stability test for 5 months. | (Yus et al., 2019) |
| Cetostearyl alcohol & olive oil | <i>L. acidophilus</i> LA3 | Granules | <i>L. acidophilus</i> LA3 granules coated with cetostearyl alcohol and olive oil 95:5 (w/w) had higher viability after incubation in simulated gastrointestinal fluids than uncoated probiotics. In addition, the coated granules showed no cells released in the simulated gastric fluid, but they were detected afterward in the simulated intestinal fluid. | (Jacobsen et al., 2021) |
| Alginate & Ca-EDTA | <i>L. plantarum</i> | Microemulsion | The microemulsion of <i>Lactobacillus plantarum</i> transforms into an aqueous form under neutral pH conditions. This provides a colon-targeted release carrier for probiotics with functional protection. | (Qin et al., 2021) |
| Pectin, Eudragit L100 & Eudragit S100 | <i>L. acidophilus</i> & <i>B. longum</i> | Tablet | The tablet dissolution study showed cumulative drug releases of around 39% to 43% in the pH 1.2 medium for two hours and up to 60% of cumulative drug releases in the pH 7.2 medium for 3 hours. In phosphate buffer medium at pH 6.8, the cumulative drug release obtained of around 98.81% to 105.08% after 7 hours. | (Sagita et al., 2022) |
| Sodium Alginate, Chitosan & Whey protein | <i>Bifidobacterium bifidum</i> | Microbeads | Chitosan and alginate-based microbeads containing probiotics were double-coated with whey protein. Microbeads provided higher survival of probiotic cells when incubated in the simulated gastric fluid and higher viability when incubated in the simulated intestinal fluids. | (Iqbal et al., 2019) |
| Sodium Alginate, Chitosan, Xanthan gum, Eudragit L100 & Eudragit S100 | <i>L. acidophilus</i> , <i>Bifidobacterium longum</i> & dexamethasone | Tablet | The coated tablet with chitosan, xanthan gum, and sodium alginate showed that the presence of probiotics in the core tablet did not affect dexamethasone release in the colon. | (Iswandana et al., 2023) |

Encapsulation methods, such as extrusion and interfacial emulsion/polymerization, can expose probiotic cells to adverse conditions during the formulation process. This can result in a decrease in the number of viable cells in the final product due to exposure to high temperatures or solvents. However, using the dry polymer powder coating technique can effectively encapsulate probiotic cells, reducing their contact with solvents and minimizing the risk of overheating. Thus, this technique is a preferable option for encapsulating probiotics.

The use of pellets for encapsulating probiotic cells has been extensively explored recently. A previous study reported that *L. acidophilus* and *B. animalis* were successfully encapsulated into pellet forms using hydroxypropyl methylcellulose acetate succinate (HPMCAS) as the coating polymer. The results showed that probiotic cells loaded in pellets had significantly higher viability when passed in acidic media and during storage compared to the free cells as well as the marketed products formulated with conventional enteric coating techniques (Park et al., 2015). Another research produced pellets containing probiotic cells by direct compression method and then coated with a combination of HPMCAS polymer and sodium alginate. The evaluation showed that the viability of the probiotics contained in the pellets increased significantly by 10^5 - 10^6 fold, compared to the free cells. The dissolution test results indicated that probiotic-containing pellets deliver probiotics to specific locations in the colon (Lone et al., 2013). Table 1 shows other examples of pellet dosage forms used for colonic encapsulation and targeting of probiotic cells.

3. Tablets

Encapsulating probiotics in tablets increases their viability and stability, with advantages including good acceptability, ease of production and administration, and storage stability. It has been reported that the compression forces and proportion of matrix-forming excipients in tablets can impact probiotic cells, disintegration, tensile strength, and cell viability. Tablets that are produced under high compression forces generally demonstrate higher cell viability. However, adverse effects can occur when the compression force exceeds 9.8 kN, as mentioned by E Silva et al. in 2013. There are two common strategies for making tablets with probiotics. One approach is to load the probiotic cells into the tablet particles, while the other is to directly compress the probiotic cells with the matrix polymers. In the direct compression method, the probiotic cells are compacted with other components to form a matrix. The resulting tablets are more stable against gastric fluids and during storage, which helps to preserve the probiotics' effectiveness.

Tablet matrix-forming polymer was used to achieve targeted delivery of probiotics, as indicated in Table 1.

For example, *Lactobacillus acidophilus* tablets were made using direct compression with microcrystalline cellulose and sodium alginate as the main excipients. The probiotic cell count was evaluated before and after compression and exposure to acidic pH 2 media. Storage stability test of tablets containing probiotics was also carried out for 3 months at 4°C. The results showed that the formulation using microcrystalline cellulose, sodium alginate, cellulose acetate phthalate, and hydroxy propyl methyl cellulose produced the highest viability after being compressed at 90.37%. Increasing the amount of microcrystalline cellulose in the tablet formulation can increase the viability of probiotics against the compression process. The tablet formulation containing xanthan showed the highest cell survival, around 72.51%, against acid exposure. A 3.97 log decrease in free cells and a 1 log decrease in probiotic tablet cells were observed after 3 months of storage. This tablet's formula and preparation allow for the maximum release of viable cells in the intestine or colon and guarantee stability until consumption (Mirzaeei & Tagheh, 2017).

Manufacturing tablets containing probiotics using a combination of two different systems can further increase the viability and stability of probiotic cells. For example, encapsulation of probiotics in tablets containing microparticles has been proposed for colon-targeted delivery of *Lactobacillus paracasei* L26. The probiotic cells were first encapsulated into whey protein microparticles and then compressed using cellulose acetate phthalate and croscarmellose sodium into tablets. These tablets demonstrated excellent probiotic cell stability in simulated gastric fluid and provided targeted delivery of *L. paracasei* L26 cells to the colon (E Silva et al., 2013). Another study was carried out to develop multi-unit tablets which are evaluated for their stability against exposure to acid, stability during storage, as well as the *in vivo* tests on the intestinal protective effect. First, pellets were produced by encapsulating probiotics (*L. acidophilus*) into hydroxypropyl methylcellulose acetate succinate using a dry powder coating technique. Then, the pellets were optimized, and they were compacted into tablets using the direct compression method. The results showed a significant increase in stability observed under storage conditions for six months. The probiotics in multi-unit tablets were found to be more resistant to acidic pH compared to the pellet dosage forms. *In vivo* studies in mice showed that repeated administration of these multi-unit tablets provided a better gut protective effect than free cells of probiotics or marketed products (Park et al., 2016). Therefore, tablets are a promising alternative for maintaining the viability of the probiotic cells and provide specific release of probiotics in the colon. Figure 2 illustrates the shape of several dosage forms that can be utilized for colon-targeted delivery of probiotics.

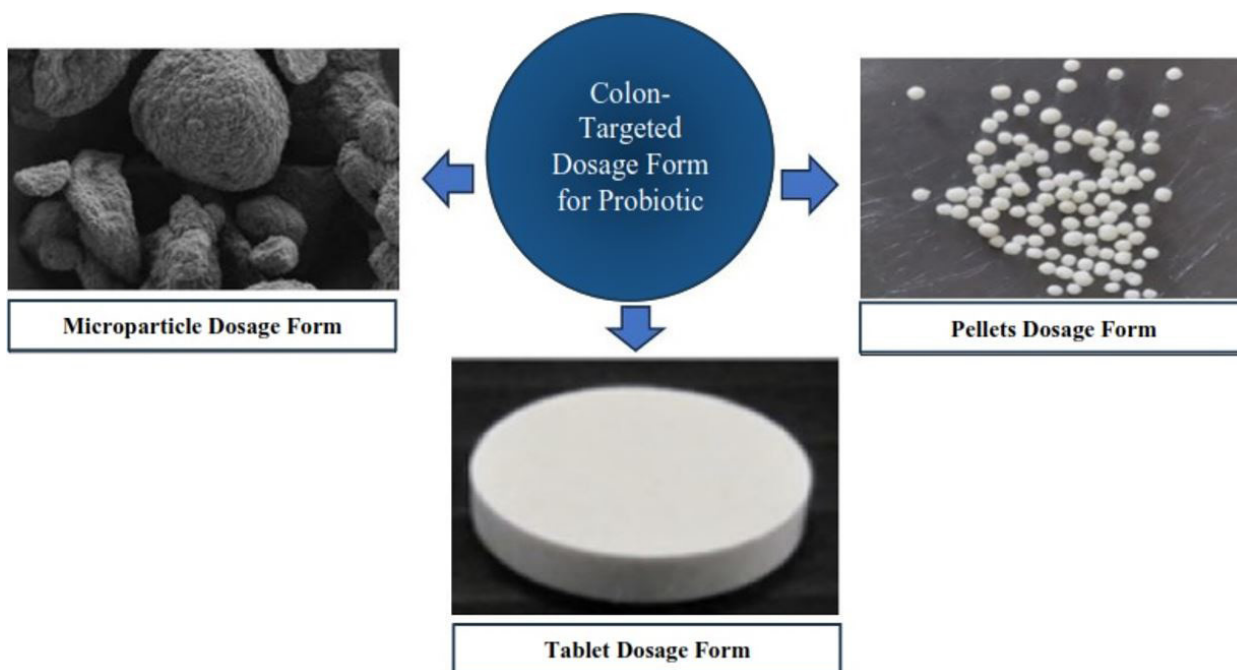


Figure 2. Colon-targeted dosage forms for probiotics (Poletto et al., 2019; Hadzieva et al., 2019 with modification)

Future Perspectives

Colon-targeted delivery systems have been extensively studied and found to be effective in releasing active compounds in the colon for medicinal purposes. However, the number of studies that have applied this system in the delivery of probiotics is still limited due to the challenges faced in handling contaminants and environmental conditions during the process. Probiotics are widely used as a supportive therapy to provide desired benefits for the body. Therefore, there is a need to develop controlled release systems, mechanisms, and suitable dosage forms for probiotic delivery to enhance their physiological benefits and protect them in the gastrointestinal tract.

To achieve colon-targeted delivery via the oral route, various approaches have been devised such as time-dependent systems, pH-sensitive polymer coatings, osmotic pressure-dependent systems, and colonic microflora enzyme-activated systems, each with its own limitations (Prudhviraj et al., 2015; Vass et al., 2019; M. Wang et al., 2021). In addition, various dosage forms have been developed based on the system and evaluated for their potential in targeting the colon. However, a system that relies on only one release mechanism cannot provide drug release at a specific site in the colon. When mesalamine tablets (Asacol[®]) coated with Eudragit[®] S, a pH-sensitive polymer, were administered to healthy volunteers, only 55% of them found mesalamine tablets or fragments in their stool samples (Shahdadi Sardo et al., 2019). Combining several release mechanisms can be

a solution, such as the combination of pH-sensitive and microbial-triggered mechanisms or combining multiple dosage forms such as tablets containing microparticles to deliver probiotics to specific areas in the large intestine (E Silva et al., 2013; Singh et al., 2021; H. Wang et al., 2016). The combination of three polymer systems, such as time-controlled polymers, pH-sensitive polymers, and separate biodegradable polymers, has good potential for colon delivery based on *in vitro* testing (Iswandana et al., 2022).

The next challenge is related to the selection of a suitable polymer. For the oral administration, the polymer must be eliminated from the body without causing any adverse effects and should be compatible with probiotics. Natural polymers such as proteins and polysaccharides show potential for use as excipients in colon-targeted formulations. However, the production of such formulations often requires the use of synthetic solvents and polymers as excipients. Therefore, it is important to investigate the toxicity of residual synthetic solvents and polymers, as well as their pharmacokinetics and pharmacodynamics in the body. Matters related to the encapsulation efficiency and release of probiotics from dosage forms should be investigated, as well as the impact of excipients on the target release site.

Combining two or more release mechanisms or dosage forms can be considered for future studies in order to provide more specific release in the colon. It is important to develop a probiotic encapsulation technique that does

not involve heat and minimizes exposure to oxygen. This will widen the range of probiotic types that can be used, making it possible to encapsulate anaerobic bacteria which are mostly the normal bacteria found in the human body. Encapsulation of anaerobic bacteria is also crucial as it will allow the use of a wider range of probiotics in the development of new products.

In the future, researchers are expected to explore the potential benefits of using a combination of two or more types of bacteria as probiotics to treat intestinal flora imbalance conditions. It has been reported that using multiple types of bacteria provides better health benefits compared to relying on a single type of bacteria. Additionally, there is a need to develop colon-targeted preparations that contain a combination of drug compounds and probiotics. This development is crucial as probiotics also aid in the recovery of colon-related diseases.

In vitro tests for colon-targeted products containing probiotics should be improved to accurately reflect the state of the gastrointestinal tract. Currently, the dissolution test results from probiotic preparations are still limited using conventional experimental conditions. To obtain more accurate dissolution test results, the addition of caecum, normal flora bacteria, enzymes, and minimal oxygen conditions should be considered. The difference in dissolution test conditions significantly affects the release profile of colon-targeted preparations. There is also a need to explore more polymers to produce specific releases in the colon since there are still limited types of polymers used in the current studies. The exploration should not be limited only to the type of polymers used but also to optimize the viability of probiotic cells in extreme conditions and provide specific release in the colon below the physiological conditions.

CONCLUSION

The development of colon-targeted dosage forms for probiotic delivery is still limited to conventional forms such as tablets, microparticles, and pellets. In addition, the main excipients used in probiotic formulations are still limited to ones providing one release mechanism to produce the targeted release in the colon. Further development is required to explore the potential combination of different dosage forms and release systems to provide specific protection and release of probiotics in the colon.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare regarding this manuscript.

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