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A Review on Enhancing Gut Health in Poultry: Probiotic Stability, Stress Management, and Encapsulation Strategies

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Abstract

The gut serves in the digestion of foods, the absorption of nutrients, and the maintenance of the host's health. Intestinal flora maintains a healthy gut by interacting with intestinal cells and inhibiting pathogens from adhering to the gut wall. Probiotics are widely used to regulate intestinal microflora, prevent and treat intestinal disorders,, and promote growth by replacing antibiotics in poultry. The current paper focuses on the effects of probiotics on gut health in general and stress factors that affect probiotic survivability from handling to the host animal's distal intestinal tract. We also go through the various ways of dealing with these stressful factors and methods adopted for industrial use. The use of encapsulation to preserve probiotics has been proven to be effective. The encapsulation strategy directly benefits stability by providing a physical barrier to safeguard them from unfavorable environments. Probiotics have been encapsulated using a variety of approaches. Here, we also discuss the effects of encapsulation on probiotic stability during different stages from processing to animal gut. Choosing the appropriate encapsulating process and encapsulating material during is crucial for producing the best microcapsule as an additive for animal feed, which ultimately improves the animal's intestinal health.

Introduction

Intestinal health and gut microbiota

The gastrointestinal tract (GIT) is a system of organs within multicellular creatures that not only digests and absorbs nutrients but also protects against diseases and toxins (Jha *et al*., 2019). Gut health involves the stability and function of bacteria throughout the gastrointestinal tract, requiring a holistic approach that includes nutrient-rich diets, mucosal integrity, a balanced microbial community, and a well-regulated immune system to maintain homeostasis and overall well-being (Bischoff, 2011; Jha *et al*., 2019; Zheng *et al*., 2020). The balance in the intestinal microenvironment leads to a healthy body but could lead to dysbiosis if there is an imbalance in the intestinal microflora. The delicate equilibrium necessary for the digestive system's efficiency is sustained by interactions among the intestinal mucus, host epithelial cells, gut-associated lymphoid tissue (GALT), and microbiota and absorption capacity (Jha *et al*., 2019; Okumura & Takeda, 2017). The microbiome's diversity is related to the health of the host's intestine. The enteric microbiome, which inhabits the gastrointestinal system, plays a vital role in nutrition and drug metabolism, detoxification, and producing essential compounds such as fatty acids, amino acids, and vitamins. Additionally, it safeguards against pathogens by competing for specific resources, influencing pathogen behavior, and impacting host gene expression and immune responses through the release of proteins and short-chain fatty acids (Fay *et al*., 2017; Jandhyala *et al*., 2015; Turner, 2018). Thus, gut health describes the function and balance of the intestinal microenvironment, majorly enteric microbiome. Furthermore, it has recently been discovered that changes in gut microbial ecosystems might contribute to immunological dysregulation and autoimmune diseases (Fay *et al*., 2017; Zheng *et al*., 2020). Numerous factors influence the gut microbiota composition, including host genotype, age, diet, localized inflammation, antibiotic use, and pathogenic organism direct invasion. Several diseases

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and conditions have been associated with alterations in the gut microbiota.

Modulation of gut microbiota

Antibiotics are harmful in two ways: they kill both pathogenic and beneficial microorganisms indiscriminately, resulting in dysbiosis and the development of non-beneficial or harmful microorganisms (Hasan & Yang, 2019). Antibiotics have long been recognized to shift the gut microbiota into temporarily quasi-stable or alternate-stable states, allowing it to become more tolerant to externalities. A decrease in diversity, a reduction of certain key species, alterations in metabolic capacity, and diminished colonization resistance against invading pathogens are all signs of post-antibiotic dysbiosis (Lange *et al*., 2016). Numerous antibiotics have served as growth promoters in farm animals, bolstering feed conversion, animal and poultry growth, and lowering morbidity and mortality linked to clinical and subclinical diseases (Butaye *et al*., 2003). However, these activities led to the spread of drug-resistant infections in livestock and humans, posing a serious public health risk. Similarly, it also showed several negative effects on gut microbiota and altered several metabolic activities in animals. Due to these effects, several alternatives have been used in poultry health to improve overall health (Gadde *et al*., 2017; Gyawali *et al*., 2021).

Supplementation of prebiotics (Yue *et al*., 2020), a mixture of oligosaccharides and dietary fiber (Cheng *et al*., 2017), traditional Chinese medicine, fecal microbiota transplantation (FMT), and other techniques have been proposed to alter the composition of the microbial community (Hasan & Yang, 2019; Yue *et al*., 2020). They are e effective in preventing and treating diseases and causing significant changes in the activity or structure of the gut microbiota, which benefit the host. However, they led to several complications. Digestive enzymes may break down prebiotics and can be absorbed by the upper digestive tract. In addition, prebiotics may also be susceptible to gastric acid (Hasan & Yang, 2019; Quraishi *et al*., 2017). The effect of FMT on the host immune system, on the other hand, is complex, and its adoption has resulted in GI irritation and associated problems. Probiotics have shown promising health benefits in humans and animals by manipulating gut microbiota. It has also shown improvement in feed utilization efficiency , increased growth performance, and reduced diarrhea occurrence in livestock and poultry (Jha *et al*., 2020; Khan & Naz, 2013; Plaza-Diaz *et al*., 2019).

Probiotics impact on gut health

The concept of probiotics is used to name those bacteria associated with beneficial effects in humans and animals. Probiotics, also called direct-fed microbials (DFMs), are live microbes including bacteria, fungi or yeast that impart beneficial effects on host health when supplemented in a definite quantity. In 2002, Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotics as live microorganisms that confer a health benefit on the host when administered in adequate amounts (FAO/WHO, 2002), which is widely accepted and adopted to date. Probiotics comprise several species of bacteria such as *Lactobacillus, Bacillus, Enterococcus, Bifidobacterium, Streptococcus, and Lactococcus* and yeasts like *Saccharomyces.* Several studies have been conducted to find their effects on animal health and their growth performance. (Gadde *et al*., 2017). The health benefits of probiotics on gut health are shown in Figure 1. Nevertheless, comprehensive studies have shown that probiotics are an essential alternatives to antibiotics due to their valuable activities on a host. Therefore, it is essential to understand how probiotics affectgrowth performance, digestive efficiency, host immunity,gut histomorphology, and microbiome.

Several mechanisms of probiotic actions are outlined in Figure 1., probiotics impart health benefits to the host by inhibiting pathogens directly or by competitive exclusion of pathogens, producing bacteriocins, stimulating the immune system, and so on (Lee *et al*., 2010; Plaza-Diaz *et al*., 2019). Competitive exclusion refers to avoiding the entrance of pathogens by blocking the cellular receptors on the luminal surface of epithelial cells that allow probiotic bacteria to stick to the intestinal cells (Callaway *et al*., 2013; Lee *et al*., 2010). In addition, beneficial microbes utilize limited sources and nutrients by competing with pathogenic bacteria and limiting the sources to pathogens that inhibit the growth of pathogenic bacteria (Ajuwon, 2016; Lee *et al*., 2010). Probiotic bacteria are also responsible for producing substances like H_2O_2 that also inhibit the growth of pathogens. Similarly, the production of organic acid and volatile fatty acid (VFA) by them lowers the pH of the gut that suppresses the growth of harmful bacteria (Erttmann & Gekara, 2019; Plaza-Diaz *et al*., 2019). Furthermore, probiotic species interact with epithelial cells and lymphocytes to show immunomodulatory effects. It also helps to raise the humoral and cellular immune response, which is accomplished through the higher production of T-lymphocytes, CD+ cells, antibody production, natural killer, and macrophage (NK) cells, and expression of anti-inflammatory cytokines, thereby stimulating the immune system of birds (Khan & Naz, 2013; Maldonado Galdeano *et al*., 2019). Additionally, probiotics stimulate epithelial barrier functions and up-regulate mucous production, which helps maintain homeostasis in the body. The secretion of mucin avoids the adhesion of pathogenic bacteria on intestinal cells (Plaza-Diaz *et al*., 2019).

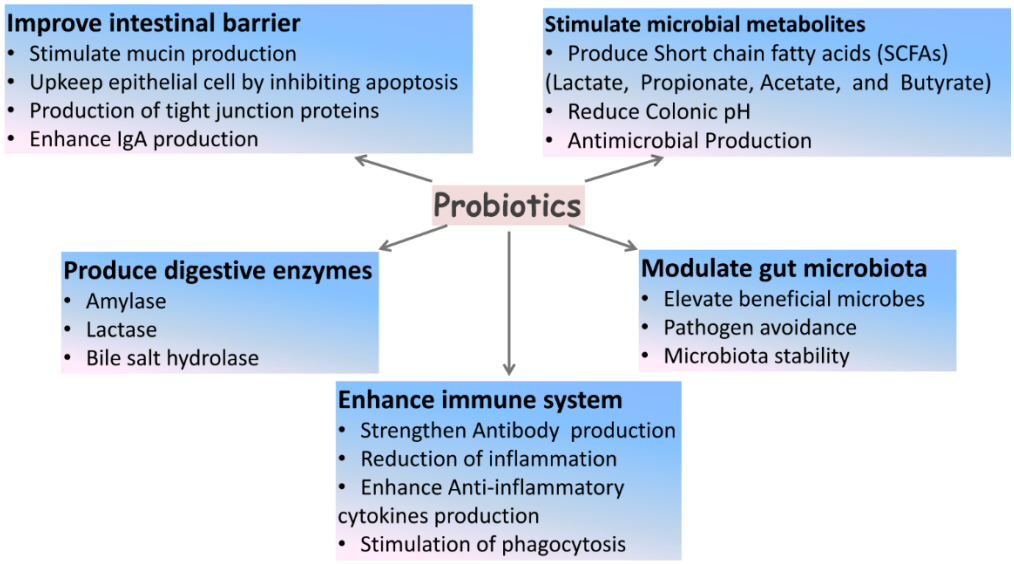

Figure 1. Health benefits of Probiotics on animal health.

In summary, major health benefits attributed to probiotics include improvement of gastrointestinal microflora, immune system enhancement, treatment of diseases and enhancement of metabolism. However, their impact was not consistent among the several studies. The inconsistency of the result could be attributed to variations in the method of administration, dose and nature of supplemented strain and their stability in the intestinal environment (lower pH, bile acid, and enzymatic activity), the difference in the physiological state of the bird, and even variation in altitude (Huyghebaert *et al*., 2011; Kalia *et al*., 2017).

Factors affecting probiotic survivability

Probiotic strains are exposed to several environmental stresses from production to ingestion. The major challenges and stress faced by probiotics are described here and given in Figure 2.

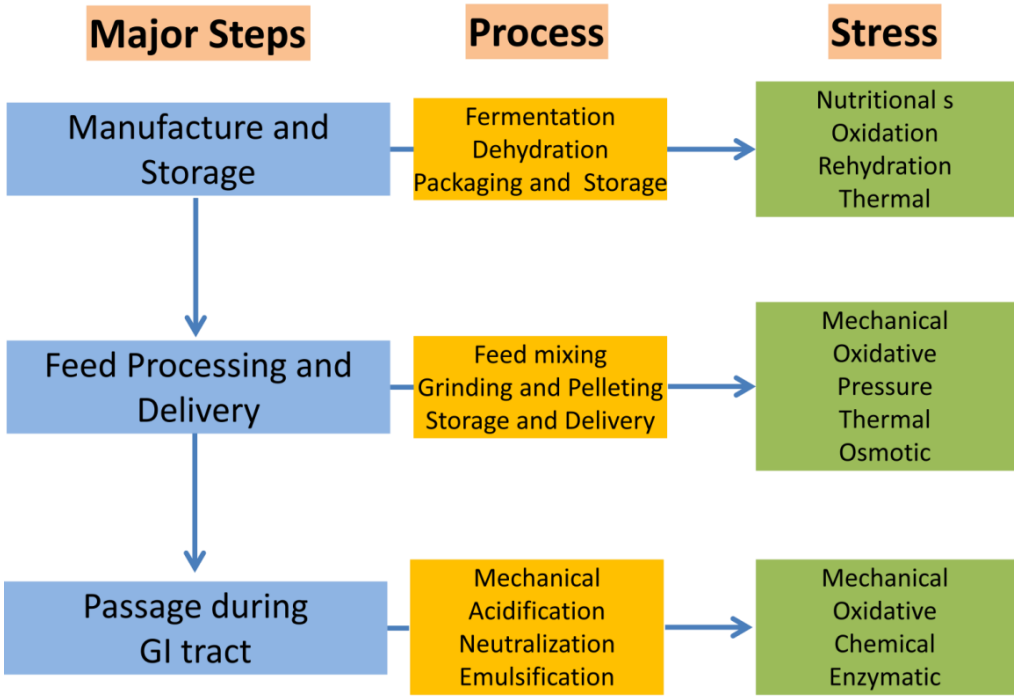

Figure 2. Probiotics used in animal feed encounter various challenges, from manufacture to ingestion.

Fermentation

For commercial production, probiotic strains should initially be selected from those with health benefits by protecting the host against pathogens. Similarly, it should withstand the stresses of manufacturing, processing, transportation, and storage. In addition, probiotics should also potentially combat the harsh gastrointestinal environment, which includes low pH and bile acid (Roos & Livney, 2016). Probiotic species chosen for commercial production must be fermented in larger numbers, which is costly and time-consuming.

Frozen seed stock, comprising single probiotic strains free of contaminants, is transferred to the main fermenter for fermentation. Growth, stability, activity, drying, and subsequent storage of probiotic strains throughout production can all significantly impact probiotic viability, depending on the media composition, growing circumstances, and type of substrate chosen for fermentation. Water, nitrogen sources, carbohydrates, salts, and micronutrients are the key parts of fermentation process (Anandharaj *et al*., 2017; Bajagai *et al*., 2010; Fenster *et al*., 2019). Instead of vitamins, glucose, salt, amino acid, and peptide size distribution, the presence or absence of other unidentified or less apparent components significantly impacts strain performance. Interestingly, within the same species, there is a wide range of sensitivities and responsiveness to the manufacturing process, which influences performance (Fenster *et al*., 2019). After meticulous fermentation, the cells undergo concentration through centrifugation, separating them from the spent media. Commercial-scale centrifugation for cell separation from the spent media is time-consuming, often taking hours due to the large cell volume, unlike laboratory-scale processes involving smaller volumes that can be completed in minutes. This difference in scale leads to varying stresses on the cells, typically involving heat and shear stress (Crittenden, 2008; Fenster *et al*., 2019).

Drying

The probiotic species are then dehydrated to make them stable for long-term use. After centrifugation, the culture media is replaced with dehydration media and the survivability of probiotics is dependent on probiotic species, dehydration method, dehydration media, and drying period utilized throughout the procedure (Golowczyc *et al*., 2011). Dehydration results in mechanical stress, changes the microbial cellular structure, or death as the large quantity of water is removed during the process. On the other hand, the encounter of cell surfaces with air or oxygen molecules causes the production and intracellular accumulation of reactive oxygen species, resulting in damage to cell lipid, protein, and nucleic acid (Iaconelli *et al*., 2015; Lemetais *et al*., 2012). Spray drying and freeze drying are the most frequent methods for probiotics and bacteria respond differently to these drying processes in terms of viability and functionality (Iaconelli *et al*., 2015). Various challenges, including thermal stress, dehydration, shear stress, osmotic, and oxidative stress, are prevalent during spray drying, resulting in probiotic inactivation and loss of viability. Similarly, during freeze drying, the formation of ice crystals causes osmotic and chemical changes in probiotics (Anandharaj *et al*., 2017; Fenster *et al*., 2019; Iaconelli *et al*., 2015). Due to outlet temperature, the strain used during spray drying, or freezing speed and cold stress in freeze-drying, the survival of several microbial strains could be reduced by up to 80% or 1 to 2 log reduction colony-forming units (Lian *et al*., 2002; Ranadheera *et al*., 2015; Zhao & Zhang, 2005). Because of the aforementioned considerations, choosing a suitable drying technique based on the features and purpose of probiotic strains is critical to maintaining bacterial viability. Once removed from the dryer, the product grins into a powder with a specific predetermined particle size and density. This milled material can then be mixed with other functional ingredients if required. This process can create mechanical stress and cell damage (Fenster *et al*., 2019).

Storage

Several factors affect the storage of dried powdered probiotics, including temperature, relative humidity (RH), oxygen, and packaging. Depending on their nature, many bacteria strains require a storage temperature of 4 or lower. Bacterial viability may be reduced due to the higher temperature (Cabello-Olmo *et al*., 2020; Mortazavian *et al*., 2006; Rerksuppaphol & Rerksuppaphol, 2010). Similarly, higher RH leads to reabsorption of water and loss of the viability of cells during long-term storage. Thus, lower RHhas been shown to improve the survival of probiotics such as lactic acid bacteria (LAB) (Min *et al*., 2017). Molecular oxygen is harmful to probiotic bacteria. Anaerobic strains of bacteria need to convert the reactive oxygen to non-toxic molecules to reduce the risk of death from oxidative damage (Ahn *et al*., 2001; Talwalkar & Kailasapathy, 2004). In anaerobic bacteria, redox reactions are modulated by pyridine nucleotides. Some bacteria, like lactic acid bacteria (LAB), consist of some O_2-consuming enzymes, including NADH oxidases (NOX) and pyruvate oxidase (POX). These enzymes reduce molecular oxygen O_2 to form H_2O_2 or H_2O (Feng & Wang, 2020; Kang *et al*., 2013). The incomplete reduction of molecular oxygen by enzymes is removed by the increase of superoxide dismutase (SOD) and glutathione (GSH) reductase activities enhancing oxidative stress tolerance. Different probiotic species have various levels of oxygen tolerance capacity

(Condon, 1987). Moreover, proper packaging could be the solution to reduce the environmental stress during storage. Materials used, permeability, and technique during packaging influence the survivability, which acts as a barrier between the external environment like oxygen and humidity, and with probiotic bacteria (Cabello-Olmo *et al*., 2020; Mizielińska *et al*., 2017). Thus, refrigeration, minimization of oxygen exposure, and regulation of environmental light by manipulating packaging material during storage could enhance the viability of bacteria.

Feed processing

It is a very challenging job to maintain the survivability of probiotic strain during feed processing. Several procedures are carried out to improve the nutritional value of food, which can remarkably reduce the survivability of probiotics. While mixing additives may not directly impact survivability, grinding might be harmful. During the grinding, heat, pressure, and moisture could reduce the viability if proper care is not taken (Follonier *et al*., 2012)

Pelleting, which improves feed palatability, is another critical stage that can affect the probiotic supplement in food-producing animals. In pelleting, small particles are combined to form a pellet by combining moisture, pressure, and heat; synergetically, this could adversely affect viability. Pelleting improves digestibility and nutrient intake and reduces feed wastage in animals (Amerah *et al*., 2013). Moreover, the pressure might reach an extreme level that could reduce viability. It is outlined that pressure more than 20 MPa and temperature greater than 50 °C affects the integrity of the bacterial cell and enzymatic reaction resulting in lower viability of probiotics during food processing (Follonier *et al*., 2012; Tripathi & Giri, 2014). After the completion of food processing, feed is transported and stored at ambient temperature for a longer time. But the change of season leads to variation in temperature. Moreover, the probiotics are also exposed to conditions like changes in RH or temperature of stored area or barn. It is reported that the storage of feed at a lower temperature around 4 °C could maintain the viability of probiotics as food is stored for a few days to months, as explained earlier in storage.

Gastrointestinal tract

After surviving from the manufacturing to storage phase, probiotic strains must be sustained during gastric transit so that the viable probiotic bacteria reach the distal gut for colonization and impart health benefits to the host. Harsh condition in the GI tract and colonization resistance due to commensal bacteria reduces viable probiotic bacteria due to which health benefits of probiotic bacteria are lessened (Chavarri *et al*., 2012; Dodoo *et al*., 2017; Yoha *et al*., 2022). After oral administration of probiotics, challenges encountered in the GI tract of monogastric animals include low pH, bile acid, pancreatic enzymes, antimicrobial peptides and so on that impact the survivability of probiotics. Poultry and swine differ in digestive tract physiology to some extent. In poultry, the crop is used to store and wet feed. Similarly, the proventriculus and gizzard function as the true stomach compartments. Hydrochloric acid (HCl) and pepsinogen are secreted in the proventriculus. Later, through muscular movement, these secretions mix with the contents of the gizzard. However, gizzards also have another function of grinding feed material, as poultry birds do not possess teeth. Moreover, the small intestine of both poultry and swine functions similarly, as a site for the digestion and absorption of most nutrients (Jiménez-Moreno *et al*., 2009; Kiarie & Mills, 2019).

Mouth and stomach

When probiotics are ingested, feed is ground and lubricated by saliva to facilitate swallowing. To safeguard oral tissues, the antibacterial proteins within the saliva, along with immunoglobulins (salivary secretory immunoglobulin A (SIgA), immunoglobulin G (IgG), and immunoglobulin M (IgM)), and non-immunoglobulin elements (lysozyme, lactoferrin, lactoperoxidases, defensins, histatins, saliva peroxidase system, and lectin protein), collaborate (Sun *et al*., 2016). When several strains of *Lactobacillus* and *Pediococcus* were exposed to saliva in an in-vitro study, no notable cell count loss was observed. However, exposure to lysozyme for a longer time could reduce survivability (García-Ruiz *et al*., 2014).

From the mouth, Probiotics transit through the esophagus to the stomach, where they encounter a highly acidic environment due to gastric acid. Acidic conditions are exceedingly deleterious to most bacteria. However, lower pH and gastric environment are barriers for pathogens entry to the small intestine. Furthermore, the acidic environment decreases the cytoplasmic pH of the probiotics. The increase in hydrogen ions (H^+) causes a decline in the activity of glycolytic enzymes within the probiotics. This decline subsequently reduces the effectiveness of the F1F0 ATPase proton pump, which is crucial for the survival of probiotics in acidic conditions (Cotter & Hill, 2003; Yao *et al*., 2020). An in vitro study of several lactic acid bacteria (LAB), including *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, and *Lactobacillus acidophilus*, demonstrated that exposure to a pH of 2 for two hours reduced their viability by 6.01 to 7.05 log cfu/mL (Ding & Shah, 2007). Furthermore, several other adverse conditions

include ionic strength and pepsin enzymatic activity affect the viability of probiotics (Yao *et al*., 2020).

Small intestine

After ingestion, bile is secreted in the duodenum to aid fat digestion. However, bile poses a crucial challenge for probiotic survival in the small intestines due to its alkaline nature and antimicrobial properties. Bile acid can damage cellular components like RNA and DNA, cause oxidative stress, disrupt cell membranes, and ultimately affect the survivability of probiotics (Begley et al., 2005; Hamner et al., 2013). Similarly, in vivo study showed that the viability of several strains of LAB including, *L. paracasei*, *L. rhamnosus*, *B. longum*, *L. salivarius*, *L. plantarum*, and *L. acidophilus* was reduced after incubation in 3% commercial purified animal bile for 4 or 8 h? (Ding & Shah, 2007). Furthermore, pancreatic enzymes such as lipase, protease, and amylase have deleterious effects on the viability of sensitive bacterial strains (Yao *et al*., 2020).

In conclusion, maintenance of probiotic survivability from production to delivery to the distal digestive tract is complicatedand includes several challenges. In monogastric animals, including pigs and poultry, the digestive tract affects the survivability of probiotic strains. Generally, lower pH due to gastric acid in the stomach and bile salt and pancreatic enzymes in the small intestine are the most remarkable challenges for influencing bacterial survivability.

Approaches for enhancing probiotic viability

Improvement of Fermentation Technology

Adjusting the culturing conditions during fermentation helps to enhance the stability and efficacy of probiotics. To maximize the biomass output and cell viability, modifying growth media and adjusting fermentation technology is critical (Crittenden, 2008). Before the process is finalized and industrial production begins, probiotics strain variables need to be typically understood and addressed in small-scale laboratory development, ramped up in the pilot, and then scaled to the commercial level (Fenster *et al*., 2019). Unique nutrients and sensitivity of different parts of the production process of each strain must be thoroughly understood and addressed within the manufacturing process to produce high-performance probiotics (Crittenden, 2008) (Fenster *et al*., 2019). The assessment of genome, gene, and protein expression and metabolism could provide crucial strain information for determining strain-specific nutritional requirements and capabilities, ultimately improving the performance of the manufactured product. Furthermore, according to the nutritional needs of the strain, the composition of complex raw components, yeast extracts, and other complex nitrogen sources can improve strain performance and viability throughout manufacturing and downstream processing (Fenster *et al*., 2019).

Stabilization of Probiotics

During the manufacture and storage of probiotics, they encounter several stress conditions that affect their stability, including temperature, oxygen, water activity, and other microorganisms. Similarly, after their survival in food processing, cells must resist the deleterious environment during the upper GIT transit, including gastric acid. Thus, to exert health benefits on the host and deliver the probiotics to the lower GI tract with a viable cell number at a sufficient level, several innovative techniques have been developed to enhance cell viability (Alemzadeh & Oryan, 2020; Dodoo *et al*., 2017; Goderska, 2012; Yoha *et al*., 2022).

The ability of different strains to cope with diverse manufacturing and storage environments varies greatly. As a result, the initial screening and selection of naturally occurring strains with improved qualities is a primary objective for enhancing probiotic stability. Lactic acid bacteria (LAB) (e.g., *Lactococcus, Lactobacillus, Streptococcus, and Enterococcus)* and *Bifidobacterium*) have a long history of safe use and exhibit favorable impact when taken at an acceptable dosage, among the known probiotic microorganisms. Industrial strains should be tolerant of pH, water activity, and aerobic conditions encountered during the manufacturing and storage of probiotic products. Acid and bile stability and intestinal mucosal adhesion qualities are crucial factors to consider when choosing probiotic bacteria. Furthermore, they must resist enzymes found in the oral cavity, such as amylase and lysozyme. In conclusion, strains with the greatest number of functional features and, at the same time, no unfavorable traits are selected (de Melo Pereira *et al*., 2018; Tuomola *et al*., 2001).

Physical stress on bacteria may evolve physiological and genetic mechanisms to allow them to resist extreme situations to thrive, despite the fact that it causes cell inactivation and improves food stability. Interestingly, using various stress situations to promote the survivability and stability of probiotics has captivated researchers' attention (de Melo Pereira *et al*., 2018). When bacteria are pretreated with sublethal stress factors before being exposed to a more lethal environment, they acquire an adaptive stress mechanism that leads to an increase in viability compared to conditions where they are directly exposed. Adaptive stress may include higher or lower temperature, oxygen, pressure, nutrient deprivation, acid, bile salt, and other stressors that could be encountered throughout manufacturing, storage, or GIT transit (Beales, 2004; Pénicaud *et al*., 2018; Terpou *et al*., 2019). Gradual adaptation of *L.*

acidophilus NCFM strains to higher temperatures improved survival in both hot and acidic conditions (Kulkarni *et al*., 2018). Similarly, heat tolerance in *Lactobacillus* was also improved after pre-adaptation to sub-lethal treatments like heat, oxidative stress, acid, and bile salt (Ma *et al*., 2021). Furthermore, it has been reported in several studies that pre-exposure to other various stresses improves subsequent survival under acidic conditions (Terpou *et al*., 2019).

To strengthen probiotic bacteria's stability at lower pH, chemicals or UV light could be used to induce random mutagenesis (Saarela *et al*., 2011). This technique produces a new strain of bacteria with more stable sensorial characteristics. A new *Bifidobacteria* strain has recently been discovered that produces less acetic acid using the same technique. A higher level of acetic acid production during manufacturing or storage is undesirable because it may result in an unpleasant flavor (Margolles & Sánchez, 2012).

Good packaging materials and techniques also aid probiotics' survival. Anaerobes, such as Bifidobacteria, are extremely sensitive to oxygen, so packaging with low oxygen permeability allows them to survive. To improve the viability of probiotic products, glass bottles with a thicker wall than plastic bottles are recommended (Shah, 2000). Due to the high cost of glass containers, other packaging methods, such as vacuum or active packaging with the addition of oxygen barrier material, absorbents, or oxygen scavengers could be a cost-effective way to maintain viability (Tripathi & Giri, 2014).

Another approach for strengthening stability is genetic engineering. It is done by introducing foreign genes from other microbes or altering the expression of genes that already present in the strain. Overexpression of chaperone in *Lactobacillus paracasei* has been reported to improve strain stability (Desmond *et al*., 2004). Similarly, the heterologous expression of Listeria's betaine uptake system (BetL) in *L. salivarius* improved stress resistance, including increased osmo-, cryo-, baro-, and chill tolerance, as well as spray and freeze-drying resistance (Sheehan *et al*., 2006).

Several components can be added to probiotics as growth promoters (e.g., carbohydrates, vitamins, minerals, and prebiotics) or processing protectants (e.g., whey protein, glycerol) to improve stability. The presence of metabolizable sugars protects and improves the survival of LABs in an acidic environment by supplying ATP to F0F1-ATPase through glycolysis, allowing proton exclusion (Corcoran *et al*., 2005). Other protective substances such as whey protein concentrate, inulin, and whey protein hydrolysate, on the other hand, have been shown to improve probiotic viability (Terpou *et al*., 2019). Similarly, combining multiple prebiotics with probiotics can enhance probiotic viability and colonization (Fei *et al*., 2021; Succi *et al*., 2017).

Encapsulation

Encapsulation is the most studied approach for improving probiotic longevity and bioactive molecule delivery. Encapsulation could assist in sustaining probiotic viability and/or positively impact the targeted animal by providing more protection against several stressors. It is the technique of encasing or enclosing probiotic cells in a wall material or membrane to protect them from harmful factors and release them at a regulated rate under specific conditions (Misra *et al*., 2022a; Shori, 2017; Yao *et al*., 2020). Encapsulation has demonstrated its efficacy in protecting against environmental alterations, such as dehydration stress encountered during drying processes (Abd-Talib *et al*., 2013; Betoret *et al*., 2020), storage (Sousa *et al*., 2012), gastric acid (Gyawali *et al*., 2023; Shori, 2017; Tee *et al*., 2014) GI tract and/or heat challenge (Afzaal *et al*., 2020a; Lasta *et al*., 2021), and other several stressors. In addition to maintaining survivability after each challenge, encapsulation enhances storage ability, allows for the regulated release of core ingredients at a specific site in the gastrointestinal system, and preserves antioxidants, vitamins, and other small molecules (Afzaal *et al*., 2020b; Misra *et al*., 2022b). The encapsulation process is influenced by several factors, including the encapsulation method, materials used, ambient conditions, capsule qualities, strain used, and the initial cell population (Šipailienė & Petraitytė, 2018). Finding appropriate encapsulation technologies and wall materials for probiotic administration might expand the range of available probiotic strains used in the feed industry and improve the performance of existing probiotics.

Extrusion, emulsion, spray chilling, spray drying, freeze drying, electro spraying, coacervation, spray-freeze drying, and other encapsulation methods are all accessible for encapsulating probiotic cells, as shown in Figure 3. However, it is critical to consider when determining a technique that the process is not aggressive, encapsulated cells are viable, and the procedure possesses mechanical stability consistent with the application purpose (Frakolaki *et al*., 2021). Several wall materials have been used for probiotic cell encapsulation, including carbohydrates (Chitosan, alginate, starch, pectin, and -Carrageenan gum), proteins (whey, caseins, and gelatin), and lipids to form the capsule structure and provide protection for core bacteria from various obstacles (Cook *et al*., 2012; Gbassi & Vandamme, 2012; Yao *et al*., 2020). Each encapsulating substance has its distinctive aspects in terms of capsule formation, appearance, shape, and strength to microbeads. The survivability of probiotics throughout storage, processing, and GI tract environments is also influenced by the

encapsulating substance used. Any material's usefulness is determined by its capacity to form capsules, strength, and improved viability, and affordability, availability, and biocompatibility (Riaz & Masud, 2013). Moreover, alginate (a polymer produced from seaweed), and chitosan (obtained from arthropods) are the most often utilized polymers (all-natural, affordable, and biocompatible) (Chavarri *et al*., 2012). The details on different methods and techniques are well explained in earlier studies (Misra *et al*., 2022b; Šipailienė & Petraitytė, 2018; Yoha *et al*., 2022).

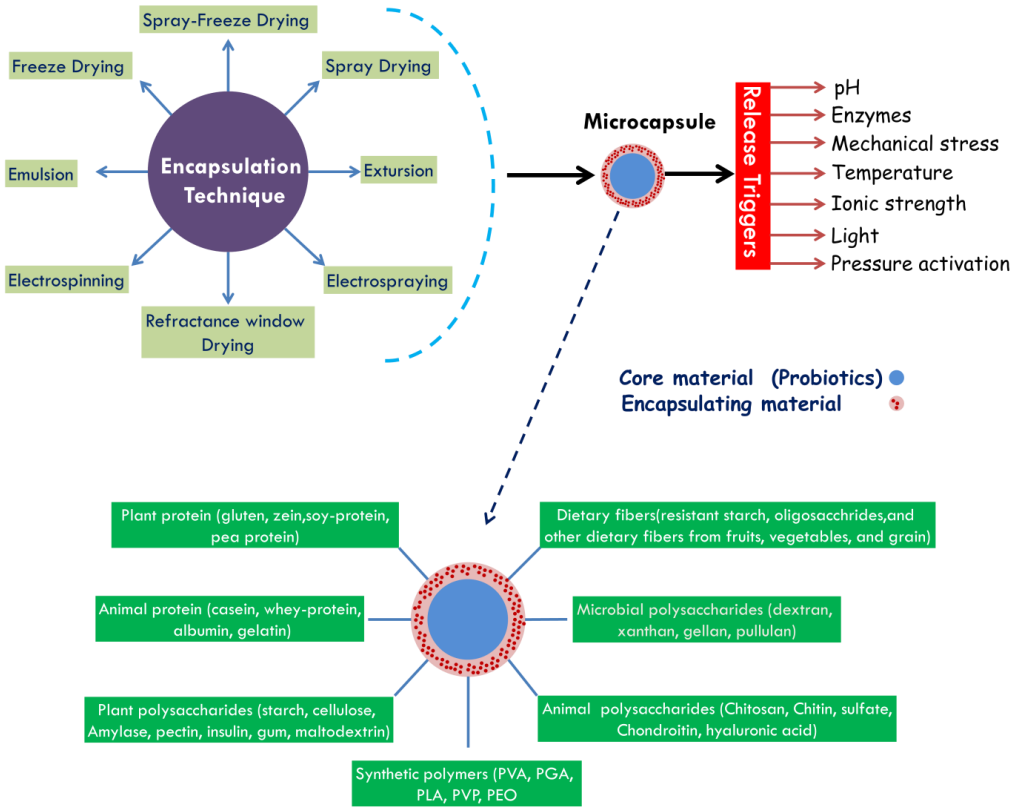

Figure 3. Overview of methods and agents for encapsulating probiotics and their triggering their release.

Effects of encapsulation on probiotic stability during feed processing

Probiotic microbes are usually dehydrated to reduce the environmental conditions necessary for storage and lengthen their shelf-life. Numerous sensitive probiotic strains are damaged by drying without any protection, thus protective material is frequently utilized to keep probiotics viable. Prior to spray drying with an output temperature ranging from 70–75 °C, two strains of *L. plantarum* (B13 and B18) were encapsulated using a combination of different protective additives such as arabic, gelatin, lecithin, and coconut oil. The vitality of *L. plantarum* B13 lowered from 1.28×10^8 to 2.1×10^6 cfu/mL, whereas the viability of *L. plantarum* B18 reduced from 3.25×10^7 to 2.15×10^7 cfu/mL (Abd-Talib *et al*., 2013). In another study, the survival of whey-encapsulated *L. reuteri* reduced from 1.6×10^9 to 2.5×10^7 cfu/g when spray-dried in a laboratory-scale spray drier with two distinct output temperatures of 55 and 65 °C (Jantzen *et al*., 2013). Similarly, following freeze-drying at -20 °C, the *Lactobacillus bacterium* strain was encapsulated in a casein-based capsule and its survivability was investigated. When encapsulated probiotics were compared to non-encapsulated or free cells, the survival rate of encapsulated probiotics was much higher (Heidebach *et al*., 2010). Thus, several studies have produced mixed outcomes regarding probiotic viability during dehydration. To obtain a definite conclusion, a thorough study of diverse formulations across a number of probiotic species should be done.

Heat is another key source of stress for probiotic microbes during feed processing. The viability of probiotics is reduced when they are exposed to higher temperatures for prolonged periods. However, multiple studies have found that encapsulating probiotic bacteria improves heat tolerance significantly. When subjected to 72, 85, and 90 °C temperatures, the encapsulated *Lactobacillus acidophilus* LA1 (by sodium alginate and starch) showed improved survivability compared to free cells (Sabikhi *et al*., 2010). Similarly, free and alginate-encapsulated *L. casei* NCDC-298 were exposed to

temperatures of 55, 60, or 65 °C for 20 minutes to test their tolerance. The vitality of free cells was dramatically reduced at 55, 60, and 65 °C, respectively, to 5.55, 4.93, and 3.98 log cfu/mL. Meanwhile, alginate encapsulation was identified to improve *Lactobacilli* survival (Mandal *et al*., 2006). In another experiment, freeze-dried alginate-based capsules coated with *L. plantarum* were heated for 5S at 75 and 90 °C. At 75 °C, free cells' vitality lowered by 3.22 log cfu/g, whereas encapsulated cells' viability reduced by 1.15 log cfu/g. Moreover, when comparing encapsulated *L. plantarum* to free cells, the same protection was observed at 90 °C for 5 s. (Fareez *et al*., 2018). *Bifidobacteium* Bb12 spray-dried with skim milk and prebiotic oligosaccharide, was challenged for 1 and 10 minutes at higher temperatures of 55, 65, and 75 °C. The free cells of *Bifidobacterium* BB-12 were susceptible to heat treatment, and their numbers reduced considerably. Conversely, encapsulation only provided significant core material protection at 55°C (Fritzen-Freire *et al*., 2013). Although the efficacy of microencapsulation depends on the probiotic strain, temperature, and duration, considerable improvements have been observed after encapsulation.

Effects of encapsulation on probiotic stability during storage

Probiotic species viability varies significantly during storage due to various factors such as strain variations, water residuals, encapsulation material and procedures, and storage temperature. *Lactobacillus* bacteria encapsulated in the sodium alginate and calcium carbonate were preserved for 150 days at -20°C, 4°C, and 25°C using a modified emulsification–internal gelation process, where 100 percent survival was found (Sánchez *et al*., 2017). After four weeks of refrigerated storage, free *L. plantarum* LAB12 lost 31.2 percent viability (from 10.4 to 7.2 log CFU g1). But, the survival of the same strain encapsulated in alginate, on the other hand, was improved by 8.5 percent (Fareez *et al*., 2018). Spray-dried microcapsules containing *Bifidobacteria* demonstrated good survivability for up to 180 days of storage at both 4 °C and -18 °C temperatures (Fritzen-Freire *et al*., 2012). Under storage circumstances, *L. rhamnosus* encapsulated with various wall materials demonstrated enhanced viability and encapsulation efficiency. *L. rhamnosus* encapsulated in a wide range of materials was placed in vacuum-sealed polypropylene bags and refrigerated at 4 °C for eight weeks. Gum arabic and gum arabic with trehalose protected *L. rhamnosus* after eight weeks of storage. Other microcapsules made of gum arabic and agave fructans, gum arabic and maltodextrin, and gum arabic and chicory inulin, however, could not sufficiently preserve *L. rhamnosus* (Barajas-Álvarez *et al*., 2022). To produce a microcapsule, *L. rhamnosus* and *L. helveticus* were encapsulated with pea protein-alginate with or without additional chitosan coating. Both microcapsules were stored in triplicate plastic barrier film bags for nine weeks under various conditions. Encapsulated probiotics maintained at 4°C had a higher overall vitality than those stored at 22°C (Varankovich *et al*., 2017). According to myriad studies, encapsulation can protect against viability loss when the storage temperature is below the ambient temperature. However, only a few research papers have been published on the benefits of storing food at/above room temperature.

Effects of encapsulation on probiotic stability during GI transit

The ultimate barrier in probiotic delivery is the administration of active encapsulated probiotic strains to the distal digestive tract. In vitro testing is widely used to investigate the efficiency of encapsulated probiotic microbes during gastrointestinal tract transit. Several studies have proven that encapsulation of probiotics with different encapsulating materials such as alginate, pectin, or chitosan provides better protection under simulated stomach acid challenges (pH as low as 1.2) and intestinal juice challenges to maintain probiotic survivability as compared to unencapsulated or free cells (Barajas-Álvarez *et al*., 2022; Varankovich *et al*., 2017; Yao *et al*., 2020; Zeashan *et al*., 2020).

At pH 2, unencapsulated or free *L. acidophilus* had the lowest viability, whereas encapsulated *L. acidophilus* with sodium alginate (SA), soy protein isolate (SPI) and sodium alginate–soy protein isolate had the highest viability. Moreover, encapsulation with SA-SPI combination showed the best outcomes regarding encapsulation efficiency and viability under simulated stomach conditions. In addition, non-encapsulated *lactobacillus* showed a substantial log reduction compared to encapsulated probiotic cells (Zeashan *et al*., 2020). Comparable results were recorded when the vitality and stability of both free and encapsulated *L. casei* were tested by simulating gastric juice (pH = 2), where non-encapsulated cells had a quick reduction in cell count when compared to encapsulated cells (Afzaal *et al*., 2020a). Similarly, after 2 h of simulated gastrointestinal juice, the average viable cells loss of *L. rhamnosus* without encapsulation was 5.25 logs CFU/g. Although microencapsulated probiotics experienced lower viability reduction compared to free probiotics, the combination of gum arabic and trehalose exhibited superior protection when exposed to gastrointestinal conditions, resulting in a final viability loss of 3.02 ± 0.03 logs CFU/g (Barajas-Álvarez *et al*., 2022). Mandal *et al*. (2006) also observed encapsulated *L. casei* survived better under low pH and high bile salt concentration conditions (Mandal *et al*., 2006).

Furthermore, when introduced to acidic conditions, the *Bifidobacterium* BB-12 strain lost viability progressively. However, compared to free cells, the microcapsules encapsulating *bifidobacteria* were sustained very well after exposure to acidic conditions in vitro (Fritzen-Freire *et al*., 2013). In our previous study, when Polyacrylate resin encapsulation was used for *L. paracasei* and exposed to simulated gastric and intestinal fluids for three hours, encapsulation increased the survival rate of probiotics (Gyawali *et al*., 2023). In other study, it was discovered that microencapsulating *B. bifidum* utilizing zein coating on alginate beads resulted in the highest viable cell count with the minimum log reduction in gastrointestinal transit and increased survivability in bile salt concentrations of 2% (w/v) (Riaz *et al*., 2019). Under simulated gastric and intestinal juices challenge tests, encapsulated *L. rhamnosus* and *L. helveticus* with pea protein-alginate also survived longer (Varankovich *et al*., 2017).

The study of encapsulated probiotic microbes in the animal gastrointestinal tract during transit has been limited. The diverse gastrointestinal system environment and the limitation of tracking mechanisms for the target encapsulated bacteria are the key reasons for these difficulties. However, some in vivo research on poultry has been conducted to determine their effects. Supplementing microencapsulated *Enterococcus fecalis* as an additive improved intestinal barrier function in chickens by enhancing intestinal morphology, immune function, and up-regulating gene expression associated with tight junction integrity in the small intestinal mucosa (Dong *et al*., 2016). Similar findings were obtained by supplementing microencapsulated *Enterococcus fecalis* CG10007 with sodium alginate, strengthening production performance and antioxidant ability. Furthermore, the microcapsule-treated groups had considerably greater counts of beneficial microorganisms such as *Lactobacillus* and *Bifidobacterium* (Han *et al*., 2013). Our in vivo study in broiler also showed that encapsulation of *L. paracasei* in polyacrylate resin imparted health benefits, improving intestinal morphology and the immune system and beneficially modulating cecal microbiota without affecting growth performance (Gyawali *et al*., 2022). Additionally, it was discovered that pre-encapsulated *Enterococcus faecalis* improves growth performance, antioxidant ability, and the number of beneficial bacteria (Zhang *et al*., 2015). Pradipta *et al*. (2019) also investigated that ingesting encapsulated probiotics enhances feed conversion ratio and villus development, which could help chickens absorb more nutrients (Pradipta *et al*., 2019). In another study, poultry were fed with encapsulated L. lactis and B. bifidum with sodium alginate and chitosan. Compared to non-capsulated bacteria, the results demonstrated a rise in total serum protein and a decrease in cholesterol (Yazhini *et al*., 2018).

In conclusion, several encapsulation techniques showed extensive protection for core probiotic bacteria during in vitro gastrointestinal challenges compared to non-encapsulated bacteria. Encapsulation, on the other hand, appeared to have the ability to increase probiotic efficiency in the distal intestinal tract in the few trials.

Conclusion

Oral administration of probiotics is both valuable and safe to improve the gut health of the animal. In the last several years, the beneficial action of probiotics and theirhealth-promoting roles have been extensively investigated. However, to produce a high-quality probiotic, proper precautions need to be established from selection to final production by developing a proper knowledge of strain, its requirement conditions including growth media, shelf-life, and storage conditions to enhance survivability. Similarly, microencapsulation is a promising technique that can protect the probiotic strains from several environmental stresses and deliver the strains to the target intestinal tract site. Moreover, encapsulation technology must be well understood to improve the viability and novel fermentation technology methods should be followed.

Conflict of interest

The author confirms no conflict of interest

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