



# Development of engineered probiotics with tailored functional properties and their application in food science

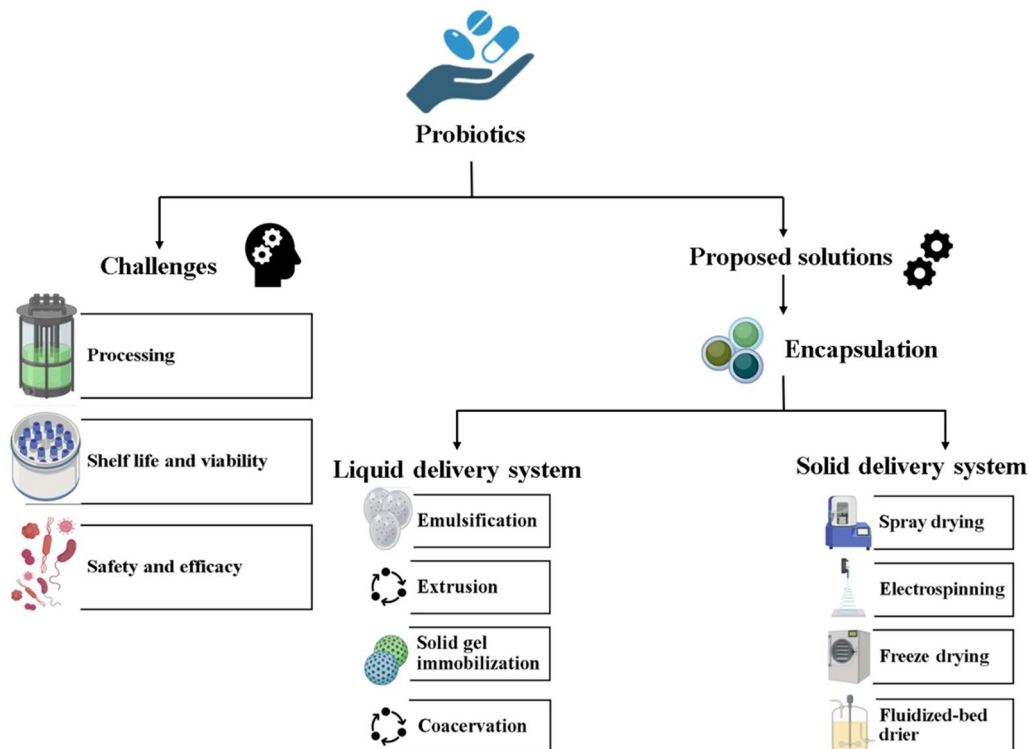
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## Abstract

The potential health benefits of probiotics may not be cognized because of the substantial curtailment in their viability during food storage and passage through the gastrointestinal system. Intestinal flora composition, and resistance against pathogens are among the health benefits associated with probiotic consumption. In the gastric environment, pH 2.0, probiotics dramatically lose their viability during the transit through the gastrointestinal system. The challenge remains to maintain cell viability until it reaches the large intestine. In extreme conditions, such as a decrease in pH or an increase in temperature, encapsulation technology can enhance the viability of probiotics. Probiotic bacterial strains can be encapsulated in a variety of ways. The methods are broadly systematized into two categories, liquid and solid delivery systems. This review emphasizes the technology used in the research and commercial sectors to encapsulate probiotic cells while keeping them alive and the food matrix used to deliver these cells to consumers.

## Graphical abstract



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**Keywords** Probiotics · Encapsulation · Stability · Viability · Solid and liquid delivery system

## Introduction

Probiotics are bacteria, moulds and yeasts, with lactic acid bacteria being the most common (Reid et al., 2019). It is typically believed that these microorganisms causes diseases and deteriorate health. The consumption of probiotics provides a number of health benefits, including improving immunity, reducing inflammation, improving digestion, making vitamins, and breaking down medications (Markowiak & Ślizewska, 2017). More than 50 genera of bacteria inhabit the human gut, particularly in the large intestine, some of which are harmful (toxins) and beneficial (synthesizing vitamins) (Zhang et al., 2015). The administration of probiotic bacteria stimulates the gut's microbiome, potentially inhibiting harmful bacteria and augmenting the body's natural defense mechanisms (Hemarajata & Versalovic, 2013). The total market for functional foods have been dominated by probiotic foods, with up to 70% of the market being probiotic foods. According to estimates, the global functional food market will reach \$ 309 Billion in 2027 and may grow by 7.5% annually between 2020 and 2027 (Tripathi & Giri, 2014).

However, the viability of probiotics continues to be a technological and marketing challenge for industries. Reduction of the viability during processing and storage still continues to be one of the substantial challenges. Encapsulating probiotics has been proposed as an efficient way to perpetuate the viability and prevent metabolic activity from deteriorating in the gastrointestinal tract (Picot & Lacroix, 2004). Encapsulation has been successfully used to ameliorate cell viability during storage of *Lactocaseibacillus paracasei* NFBC 338 by spray-drying (Desmond et al., 2002), *Lactocaseibacillus casei* NCDC-298 by emulsification (Mandal et al., 2006) and more. This review analyses techniques used for encapsulation and factors that influence the viability of cells.

## Bioengineering of probiotics

Many factors such as oxygen levels, redox potential, additives, antimicrobial compounds, and bacteriocins are found to affect the viability of cells present in probiotics during storage (Terpou et al., 2019). Biological factors include strain type, natural microflora product, enzymes produced, post acidification and various pathogenic or spoilage microorganism occurrences (Vieira da Silva et al., 2016). Physical factors include drying conditions, and temperature associated (Fenster et al., 2019). Strategies to enhance cell viability include selecting suitable strain, which serves a crucial role in improving viability.

In order to prompt cell inactivation and to intensify cell stability, physical stress is applied (temperature stress, osmotic stress, oxygen stress) (Fenster et al., 2019). The selection of proper food packaging systems can influence viability. Including packaging methods like oxygen scavengers, and vacuum packaging can significantly improve viability (Souza et al., 2012).

Encapsulation technologies are expected to enhance stability, and ensure better handling and storage of probiotic cultures. As most probiotics are of intestinal origin, they are unsuitable for growth in dairy-based media and mostly get inactivated on exposure to high heat, and acid during processing (Zielińska & Kolożyn-Krajewska, 2018). Technological challenges associated with maintaining a high number of probiotic organisms in food, the capability of the culture to retain viability in the food matrix environment, and maintenance of its characteristics during consumption are also of concern. Spray-dried and freeze-dried cultures help introduce the culture of the human gastrointestinal system. But sometimes, these technological approaches used for the preparation of cultures may affect their viability and functionality. Sometimes cell injury can also happen during the application of these technologies (Irvani et al., 2015).

Probiotic encapsulation technology has rapidly emerged in the past decade. With the help of this technology, many microorganisms have been immobilized with semipermeable materials to facilitate their delivery (Hassan et al., 2019). Despite the benefit of increased viability and shelf life, it faces many challenges, including developing microencapsulation equipment, selecting non-toxic materials for encapsulation, developing beads or capsules from polymers, and determining the appropriate mechanism of probiotic release, carrying out these are assigning their costs. One of the most important challenges is of the cost, encapsulated end products can be very costly (Aragón-Rojas et al., 2019). This is because their development demands both time and financial resources. Using natural polymers will increase the cost further, milk proteins seem more costly than carbohydrates. These techniques also require certain raw materials, which include oil and emulsifiers, in order to stabilize the capsule (Aragón-Rojas et al., 2019).

## Encapsulation of probiotics

Microencapsulation method describes the process of packing or encapsulating solid, liquid, or gaseous materials into tiny capsules that can protect active or functional materials from hazardous environment and release their

contents under certain conditions at a controlled rate (Yao et al., 2020). Within the microcapsule core are probiotics that are present and survive, and surrounding the core is a very thin but strong membrane that allows contents to enter and exit the microcapsules. A microcapsule can be designed to release active ingredients gradually under the influence of heat, solvation, diffusion, and pressure. An engineered coating can also allow microcapsules to open at specific locations on the body (Bepeyeva et al., 2017). Having developed the science of microencapsulation and advanced biomaterials, biomaterial-based microencapsulation has become a popular method for encapsulating probiotics for enhanced stability (Dafe et al., 2017). Encapsulation of probiotics with carrier material, particle size, and its advantages are represented in Table 1.

## Challenges with use of encapsulated probiotics

### Microencapsulated probiotic stability when lyophilized or spray dried

Live microorganisms are those that are beneficial to the host and can be easily administered in adequate amounts. The study analyzed the effect of various encapsulating agents on the feasibility of freeze-dried *Lactocaseibacillus casei* capsules. The viable cells were determined using the spray drying technique. They were then subjected to heat treatment at temperatures of up to 90 °C (Reid et al., 2019). The use of different encapsulating agents significantly improved the survival rate of *L. casei* Shirota when exposed to acidic conditions. Microencapsulated cells exhibited the viability of being subjected to 1% bile salts in the presence of encapsulating agents. The cells were also stable during incubation (Gul, 2017). When subjected to 2% bile, the microcapsules exhibited a decrease in size of about 1.09 log. This effect was caused by the use of spray or dried freeze methods. *L. casei* Shirota was microencapsulated with various additives to improve its survival against the stresses of the environment. This included freeze-drying and spray formulations. The viability of microcapsules was maintained at 5 log CFU/g for up to 5 log CFU/g exposure (Gul & Atalar, 2019).

### Solidness of microencapsulated microscopic organisms in gastrointestinal tract

The direction of the research was on the effects of different encapsulating agents upon growth and survival of freeze-dried *L. casei* capsules in the gastrointestinal tract. It is revealed that varying agents affected the bacteria's viability. Encapsulated cells exhibited the ability to endure the

presence of 1% bile salts. They also exhibited a stable state during incubation. When dehydrated, 2% bile microcapsules decreased in size by about 1.09 log (Castro-Cislaghi et al., 2012). The reason for the decrease in microcapsules size was due to the usage of spray or freeze methods. *L. casei* Shirota was microencapsulated with several compounds to improve its resistance to environmental stress. Freeze-drying and spray formulations were among them. Microcapsule viability was sustained at 5 log CFU/g exposure for up to 5 log CFU/g. Gul and Atala (2019) also stated that a great opportunity to improve the preservation of probiotics in severe acidic conditions in the stomach by using enzymes like rennet or TGases in the course of probiotic encapsulation in dairy-based matrices. Since then, other research has been successfully guided the improvement of feasible probiotics in regard to plant and probiotic materials (Andrade et al., 2019).

## Biopolymer as stabilizer matrix

Biopolymers are naturally produced by cells and are made up of repeated chains of monomers that are covalently linked together (Brigham, 2018). The most commonly used biopolymers for probiotic encapsulation are carbohydrates (such as alginate, carrageenan, and gums), proteins (such as whey protein, casein protein, and soy protein) and lipids (such as waxes, and fats) (Gutierrez & Alvarez, 2017). The categorized materials employed for the preparation of encapsulation are represented in Fig. 1.

### Carbohydrate-based system

Carbohydrates-based systems are proven to have thickening, stabilizing, and gelling properties. They are preferred because of their non-toxicity, biocompatibility, and biodegradability. Some of the widely used carbohydrate systems are alginate, carrageenan, chitin, starch, and gum. (Martău et al., 2019).

Alginate, an potential encapsulation material does not dissolve in an acidic environment, thereby protecting the probiotic microbe (Oberoi et al., 2021). Emulsification and complex formation can be used to create alginate microspheres. Both methods can optimize the particle size as required. Coating the nanoparticles with chitosan to make the capsule less permeable to water-soluble molecules and for a smoother surface, thereby increasing the stability of the probiotic. Studies indicate that the viability increases with an increase in the capsule size but huge beads with a size > 1 mm does not significantly improve the viability (Ephrem et al., 2018).

Carrageenan is a linear polymer formed by repeating units of  $\beta$ -D-glucopyranose and 3, 6-anhydro- $\alpha$ -D-glucopyranose

**Table 1** Encapsulation of probiotics with carrier material, particle size and its advantages

Name of the bacteria	Carrier material	Encapsulation method	Particle size	Effect/findings	References
<i>Lactobacillus acidophilus</i>	Sodium alginate, Hi-maze starch	Extrusion	70 µm	Provided better protection against the intestinal and gastric juices after they were exposed to the moist microcapsules. The moist form showed greater viability than the freeze-dried form.	de Araújo Etchepare et al. (2016)
<i>L. fermentum</i>	Alginate, oligosaccharides	Extrusion	2.34–2.71 mm	All these microcapsules were stable at simulated gastric conditions.	Liao et al. (2019)
<i>Lactococcus lactis</i>	Alginate	Extrusion	155 ± 116 µm	Showed improved resistance to environmental stress.	Yeung et al. (2016)
<i>Pediococcus pentosaceus Li05</i>	Alginate, gelatin, MgO nanoparticles	Electrostatic Microencapsulation	500 µm	Showed enhanced viability in long-time storage, heat treatment and gastrointestinal transit.	Yao et al. (2018)
<i>S. cerevisiae (KTP)</i> <i>Issatchenkia occidentalis</i>	Maltodextrin-sucrose Maltodextrin-sorbitol	Spray drying	9–10 µm	Encapsulation enhanced the survival of the yeast in bile and gastric juices	Suryabhan et al. (2019)
<i>L. paracasei</i> <i>BGP-1</i>	Alginate and alginate—shellac blend	Fluidized bed	0.71–0.86 mm	The microcapsules showed improved viability since fluidized bed drying gave the microcapsules robust structure. The alginate-shellac blend also helped by reducing the porosity of the structure. This method is promising to vehiculate probiotics in non-dairy products.	Vieira da Silva et al. (2016)
<i>L. paracasei</i> KS-199	20% (w/v) PVA; 3% (w/v) sodium alginate; strain solution (7:1:2, v/v/v)	Electrospinning—single nozzle, plate collector	842 nm	86% viability after electro spinning. This method is promising since microencapsulation increased the survival of probiotic strain in gastric conditions.	Yilmaz et al. (2020)
<i>L. acidophilus</i> 016	5% bacterial cellulose was mixed with the same volume of 5% PVA	Electrospinning—single nozzle, plate collector	576 nm	71% viability during storage at 35 °C after electro spinning	Jayani et al. (2020)
<i>L. paracasei</i>	Starch-formate/glycerol compound fibers	Coaxial electrospinning	4.45 ± 0.91 µm	This encapsulated bacteria endured the storage condition of 4 °C and RTP for 21 days.	Lancuški et al. (2017)
<i>L. plantarum</i>	Monolithic PEO and composite PEO/lyoprotectant fibers	Electrospinning	815 ± 115 nm	Enhancement of viability during electrospinning and storage	Škrlec et al. (2019)
<i>L. casei</i> , <i>L. paracasei</i> , <i>L. plantarum</i> , <i>L. acidophilus</i> , <i>B. adolescentis</i> and <i>B. Bifidum</i>	6% PVA solution with 2.5% FOS	Electrospinning—single nozzle, plate collector	410 nm	FOS addition enhanced the viability of the probiotic while electrospinning and 93% viability after electrospinning	Feng et al. (2018)

(Ephrem et al., 2018). It is of three types, namely,  $\kappa$ -carrageenan,  $\iota$ -carrageenan, and  $\lambda$ -carrageenan. An oxygen bridge present between the third and sixth carbon of D-galactose in the kappa and iota carrageenan, which makes gelation possible. Chitosan is a hydrophilic polymer with amazing muco-adhesive properties, which aids in the improvement of the release of bacteria in the gut and changes the gut microbiota through its antimicrobial activity. Alginate-chitosan system shows improved stability under stimulated GI conditions. It also increases the probiotics' survival rate in simulated gastric and intestinal conditions and reduces viability losses during freeze-drying and freezing. The drawback of using this as the coating material is that chitosan does not dissolve in pH above 5.4 because the gut's pH is greater than 5.4 (Călinoiu et al., 2019). However, studies have found that a combination of chitosan with other polymers can be utilized for a system with better stability. Another study has shown that a mixture of chitosan and whey protein, chitosan and gelatin produce capsules with increased stability (Choińska-Pulit et al., 2015). When alginate beads are coated with chitosan, the surface becomes smoother and hence less permeable to water, thereby increasing the stability (Ephrem et al., 2018).

Gellan gum and xanthan gums are anionic bacterial linear tetrasaccharides (Milivojevic et al., 2019). *L. casei* was encapsulated into gellan gum and sodium caseinate. After subjecting both the immobilized and free cells to simulated gastric fluid for 120 min, the vitality of free cells dropped by 6.1 log CFU/g whilst the vitality of immobilized cells dropped by just 3.1 log CFU/g. It is clear from this that encapsulation increased the viability of the probiotic (Shori, 2017).

### Protein-based systems

Proteins are polymers made up of peptides used as encapsulating materials due to their amphiphilic nature. Casein, whey protein, and gelatin are vastly used for encapsulating probiotics (Nguyen et al., 2015). Gelatin have been manufactured by partial hydrolysis which denatures collagen. It is preferred for encapsulation due to its thermo-reversible nature which makes it possible for gelatin to melt and become liquid when heated above its melting point and when cooled it hardens and forms gel (Fathi et al., 2018). Its benefits include non-toxicity, the excellent ability to form membranes, and biocompatibility (Agarwal et al., 2016). In order to improve its rigidity, cross-linking can be done (Skopinska-Wisniewska et al., 2021). Probiotics encapsulated with gelatin and coated with maltodextrin showed enhanced viability (Sohail et al., 2013). Recently, an experiment has been carried out in which the probiotic yeast *Kluyveromyces lactis* was encapsulated in gelatin hydrogels which were obtained by cross-linking the gelatin chemically. Hydrogels having

the highest concentrations of gelatin (7.5%, w/v) which the concentrations of cross-linking agents were found to be 3.0% and 5.0% (w/w) were selected for encapsulation. The efficiency of encapsulation was found to be 10%, and there was 50% cell viability level when subjected to simulated gastric conditions (Albadran et al., 2020).

Casein, a milk protein, is a popular encapsulating material for probiotics (Shori, 2017). Casein has excellent gelation property, initiated by unfolding of protein by denaturation followed by aggregation (Wu et al., 2021). Non-covalent bonds aid in stabilizing the structure more than covalent bonds. It is water-insoluble in nature and protects the bacteria during gastric transit (Shori, 2017). Encapsulation of *Bifidobacterium* bb12 and *Lactobacillus* F19 in casein microstructures was done by enzymatic gelation, and their resistance against dehydration during cryodesiccation and storage was tested. It was found that *Bifidobacterium* showed an increase in viability during storage upto 90 days, whereas *Lactobacillus* didn't show a substantial change in survivability in comparison to cells that were not encapsulated (Irvani et al., 2015).

Whey protein is frequently employed protein for probiotic encapsulation. It is preferred especially due to its amphoteric nature and its ability to be mixed easily with negatively charged polysaccharides. This happens when the net charge of the protein becomes positive as a result of the pH being set below the isoelectric point (Choińska-Pulit et al., 2015). Whey protein has been used to encapsulate *Lactocaseibacillus rhamnosus* strain by extrusion. 96% of bacterial cells had been incorporated into whey protein capsule successfully (Doherty et al., 2012).

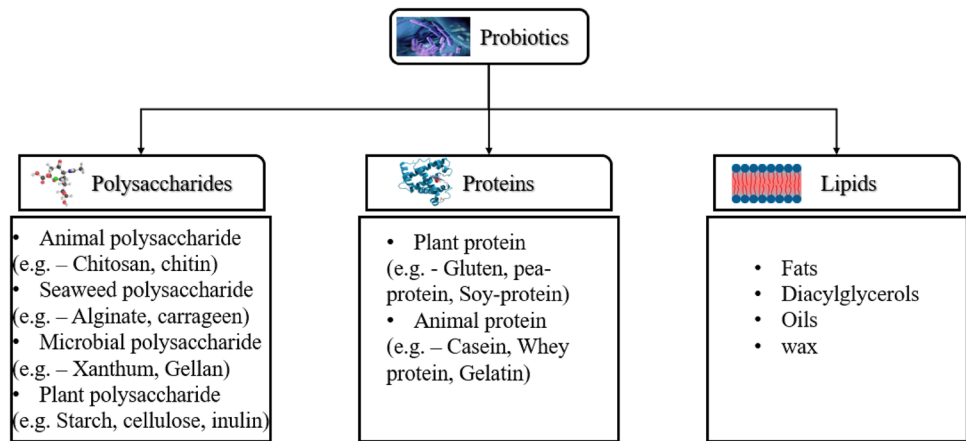
### Natural support

The non-digestible carbohydrates present in fruits acts as the foundation for encapsulating probiotics. In this context, using quince and apple pieces as support, *L. casei* cells were effectively immobilized. Immobilized biocatalysts were also used to synthesize lactic acid and probiotic additive fermented milk, whilst immobilized microbial cells regained activity after 129 days at 4 °C. Throughout the storage time, a fruity, unmistakable scent pervaded the fermented milk. *Lactocaseibacillus casei*, immobilised on fruits were also effectively employed in the manufacture of cheese containing probiotic (Aziz & Temiat, 2020).

Fruits and oats pieces were also considered to be used as support for transporting *L. casei* ATCC 393. Cell viability was examined throughout refrigerated conditions of the immobilized cells, which were utilised to make probiotic yoghurt. According to strain-specific multiplex PCR analyses, not only immobilized but free cells were also identified at sufficient quantities for conferring a probiotic effect for longer durations than needed by the dairy sector (30



**Fig. 1** Categorized materials employed for encapsulation



days) while stored at 4 °C (Bosnea et al., 2017). By adopting a vacuum impregnation process, attempts were made to merge the health benefits of probiotics with the benefits of fruits and vegetables. Encapsulated *L. salivarius* is vacuum impregnated into apple discs which were later dried at 40 °C for 24 h, and after the gastrointestinal simulation for 30 days the viability of the cells were evaluated. It was found that the dried apple discs with the encapsulated cells that showed enhanced viability than the free cells (Ester et al., 2019).

Similar research revealed that alginate micro beads were used to encapsulate *L. rhamnosus* and *Lactobacillus acidophilus* at 25 °C and 4 °C their acidification carried out in orange juice was investigated for 9 days and around 30 days, respectively (Sohail et al., 2012). Orange juice has been considered as a great probiotic carrier since it has high antioxidant ascorbic acid that has the ability to protect the oxygen sensitive bacteria. Encapsulated *L. rhamnosus* showed enhanced viability compared to *L. acidophilus*, indicating that the increase in survival depends on the species. The findings also show that even though encapsulation does not protect the sensitive probiotic cells from death, it certainly reduces acidification in orange juice (Perricone et al., 2015).

### Synthetic polymers

Synthetic polymers are not naturally produced by cells in nature. There are a lot of synthetic polymers used commercially for encapsulating probiotics today. Poly(lactide-co-glycolide) is a synthetic polymer used to encapsulate hydrophobic compounds that need time-dependent release. This is a desired encapsulating material due to its biocompatibility, biodegradability, and controlled delivery. The use of poly D, L-lactic-co-glycolic acid (PLGA) for encapsulating probiotics has its limitations, as the organic solvents used for polymer solubilizing can lead to cell damage (Asgari et al., 2020). Cellulose acetate phthalate is another synthetic polymer used as an encapsulating agent. Cellulose acetate phthalate has the property of being soluble at the pH above

6.0 but it is not soluble below the pH 5.0. This is a desired property since the biomaterials should dissolve in the gastrointestinal tract but not be soluble in the stomach (Amidon et al. 2015). Polyvinyl alcohol, a hydrophilic synthetic polymer that is widely used for encapsulating microbes. This is a desired biomaterial since it is inexpensive, non-toxic, durable and highly stable. It has been used in different eatables such as cereals, and yogurts. (Ephrem et al., 2018). Various encapsulation methods and their respective advantages and disadvantages are elaborated in Table 2.

### Application of encapsulation technologies in probiotic food production

Although many microorganisms synthesize lactic acid, such as *Enterococci*, *Streptococci*, *Lactococci*, which are utilized as probiotics strains incorporated in food matrices, and the most frequently used ones belong to *Bifidobacterium* and *Lactobacillus* genera (Sarao & Arora, 2017). To enhance the host health benefits, it is commonly believed that the minimum concentration of probiotic cells that survive in the food matrix should be around  $10^6$ – $10^7$  Colony forming unit (CFU) per gram or ml. Therefore, there is a technological barrier in preserving the viability of the microorganisms that are incorporated into the foods under the condition of processing, storage, distribution, and consumption. Various edibles have been utilized to deliver probiotics into the gut (Rodrigues et al., 2020).

Fermented foods are ideal vehicles used to deliver probiotic bacteria, especially fermented milk, as the number of proteins, lipids, and carbohydrates present in these matrix can increase the bacteria's viability. Encapsulation of probiotics is required since dairy foods are acidic and are not undersuitable atmosphere for the stabilization of the microbes. Therefore, immobilization methods can be employed to enhance the viability from the harsh environment (Alvarado-Reveles et al., 2019).

Proteins as well as essential micronutrients including zinc, iron, and vitamin B12 are abundant in meat products.

**Table 2** Various encapsulation methods and their respective advantages and disadvantages

Encapsulation method	Type	Advantages	Disadvantages	References
Emulsification	Solid	High reproducibility, ease of scale-up, and narrow size distribution	Reduced encapsulation efficiency due to removal of large volume of water from the suspension	Spyropoulos et al. (2014)
Coacervation	Solid	Can encapsulate heat sensitive ingredients	Toxic chemical agents are used and complex coacervates are unstable	Sing (2017)
Freeze-drying	Liquid	Easy to handle and transport. Long term storage is possible	Complex process which may require cryoprotectants. These are responsible for formation of ice crystals which may lower cell viability	Liu et al. (2022)
Spray-drying	Liquid	It is inexpensive and a continuous process. Mass production is possible	Loss of cell viability and can mostly be used only in aqueous solution	Leng et al. (2018)
Electrospinning	Liquid	Simple process which can mimic the native ECM structure	Toxic solvents are used and heterogeneous cell distribution is observed	Hong et al. (2019)
Fluidized bed dryer	Liquid	Lower thermal stress with rapid heating. It's a scalable operation	Strain applicability is limited	Alonso (2016)
Nanoemulsions	Liquid	Transparent and translucent, can be used in beverages, rapid absorption and good shelf life	Low stability in acidic environment, rapid release	Blanco-Padilla et al. (2014)
Nanocapsule	Solid	Increase bioavailability and biodegradability due to use of natural polymers	Purification process is necessary after synthesis	Huang et al. (2013)
Nanofibers	Solid	Large surface area and high porosity. Large scale production is possible. Can carry heat sensitive compounds	Limitation is biopolymer solubility	Okutan et al. (2014)
Solid lipid nanoparticles	Solid	Decrease in toxic side effects and increase in aqueous solubility of compound	Low encapsulation load and risk of recrystallisation	Gaur et al. (2013)

It also contains a meagre quantity of monosaturated and polysaturated fatty acids (Ekmekcioglu et al., 2018). Probiotics can be incorporated into meat in order to increase the nutritional value of meat products, simultaneously reducing the negative effects. For this, thermos-resistant encapsulated probiotics are used to increase its viability (Chavarri et al., 2012).

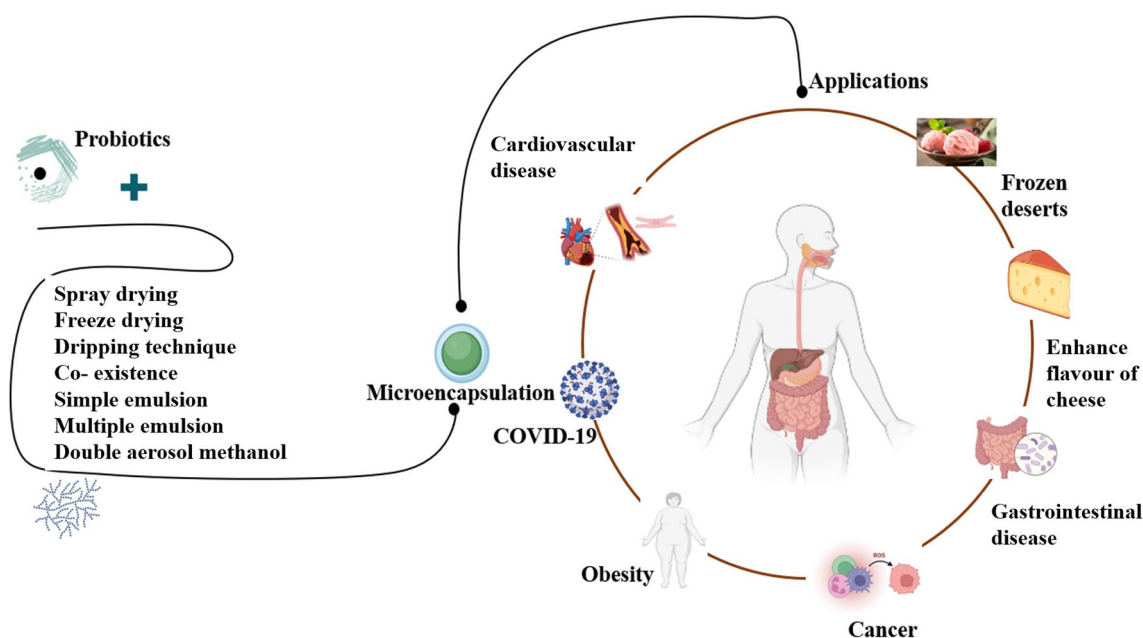
### Edible coatings as encapsulating matrices

Edible coatings are non-toxic polymers that can be utilized as encapsulating matrices for probiotics that provide a moisture and oxygen barrier (Quirós-Sauceda et al., 2014). The encapsulation provides a microenvironment that protects the core material from the outside environment. These help in extending the viability during storage and minimize the risk of contamination by pathogens and also protect the probiotic microbe from extreme environmental conditions. The method used to encapsulate depends upon the properties of the probiotic, edible coating material which are based on proteins, carbohydrates, and lipids (Pech-Canul et al., 2020). The applications of encapsulated probiotics are elaborated in Fig. 2.

Because of their outstanding mechanical and optical characteristics, proteins are regarded as excellent polymers for edible coatings and good barriers of CO<sub>2</sub> and O<sub>2</sub> (Pech-Canul et al., 2020). The lipids also make up effective edible coatings due to their low polarity, they largely obstruct moisture transfer. Usually, lipid-based films are in combination with polysaccharides for extra mechanical strength (Pech-Canul et al., 2020). The bacteria *L. rhamnosus* CECT8361 was coated with alginate enriched with inulin and oligofructose and used to coat blueberries. This edible coating increased the survival of the bacteria and enhanced the antimicrobial property of the end product, thus minimizing the quantity of *Listeria* inoculate (Bambace et al., 2019). These edible coverings have been used in a fresh-cut vegetables to increase shelf life. It has also been utilized to incorporate probiotics in meat and fish products.

### Co-encapsulation of probiotics and bioactives

Synbiotics are now defined as “a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host” by the ISAPP. The synbiotics are classified into complementary synbiotics and synergistic synbiotics. Complementary



**Fig. 2** Applications of encapsulated probiotics

synbiotics constitute prebiotics and probiotics that are independent of each other. The substrate in synergistic synbiotics is intended to be exclusively used by the probiotic bacteria that is administered along with it (Swanson et al., 2020). Data suggesting the efficiency of synbiotics on the gut microbiota is compelling. These researches revealed that symbiotic is able to inhibit harmful microbes by direct antagonism and competitive exclusion and also expedite the recovery of the health of the intestinal microbiome.

The positive effects of these co-encapsulated synbiotics depend upon their specific combination, therefore the antimicrobial activity and strain specificity has to be taken into considerations. By controlling specific gut bacteria, synbiotics assist to balance the gut microbiota, paving the way for the creation of new forms of functional foods with a more precise effects than dietary supplements or other symbiotic-rich products. Moreover, it can also help to combat multi-drug resistant microbes. Co-encapsulation is used to promote synbiotic oral administration, durability, survivability, and also tailored discharge inside the gut. Therefore, selecting the right co-encapsulation technology is important since it needs to improve the survivability of the microbe against the extreme environment of the gastrointestinal tract. The structure and composition of the encapsulating material, as well as the selection of appropriate co-encapsulation technique, determine the encapsulation effectiveness and viability of co-encapsulated probiotics (Chen et al., 2017). A few examples of co-encapsulation are given below.

Gamma-aminobutyric acid (GABA) is a natural non-proteinogenic amino acid. It functions as a bioactive inhibitor

of neurotransmission in the mammalian central nervous system. GABA is offered as supplements as it is found only in small quantities in its naturally occurring forms such as coffee, cereals, vegetables and fermented foods (Hepsonali et al., 2020). It also have anti-cancer, anti-anxiety, and anti-diabetic effects, and insufficient levels of GABA has been shown insomnia, anxiety and weaker immunity systems. Co-encapsulating GABA along with *L. plantarum* NCDC 414 can be used to improve its nutritional potential (Pandey & Mishra, 2021).

Omega-3 polyunsaturated fatty acids (PUFAs) are bioactive lipids that are naturally available in few plants, fish products, and a few vegetable oils. Omega-3 PUFAs incorporated into human diet by microencapsulation, thereby minimizing oxidative degradation, increasing stability and bioavailability (Chen et al., 2017). Furthermore, since probiotics enhance the action of PUFA and vice versa, co-encapsulating is a promising strategy.

Co-encapsulation of dietary fibres are advantageous to human health because they may change speciation diversity and size of the bacterial colonies and inhibit microbial adherence (Wu et al., 2020). Probiotics were effectively combined with several varieties of dietary fibers in microspheres, improving their shelf durability, processing resistance, and transit through the GI tract (Ying et al., 2016). Encapsulation of probiotics along with maize starch has shown improvement in viability compared to encapsulating just the probiotic alone (Etchepare et al., 2016).



## Stability of encapsulated probiotics

Although encapsulation of the probiotics increases the stability considerably, there are a lot of factors that affect its stability. The major elements that determine the stability of immobilized probiotics are high temperature, UV light, solubility, controlled release, and anti-microbial effects (Mitropoulou et al., 2013). The glass transition temperature must be measured using differential scanning calorimetry in order to determine the stability of the encapsulated probiotics. At glass transition temperature, the permeability of the coating material is low and preventing oxygen entering into the capsule and thereby preserving the probiotic bacteria (Călinoiu et al., 2019). As the deterioration due to bacterial growth and other chemical reactions is very low when stored lower than the glass transition temperature, the shelf life of the encapsulated probiotics is extended. If it is stored at a temperature greater than the glass transition temperature, the entry of oxygen accelerates various chemical reactions (Liliana et al., 2014).

One of the important things that affect the efficiency of encapsulation and its ability to release probiotics in the desired region. The controlled release and the carrier material plays a vital role (Liao et al., 2020). Alginate is considered a great polymer for encapsulating probiotics as it is stable in an acidic environment but disintegrates when introduced into alkaline conditions, and thereby releasing the microbe. But due to the pores present in the alginate beads, it might decompose into hostile environment. This can be avoided by combining alginate with other materials, such as chitosan, gelatin, and pectin (Liao et al., 2020).

The bacteria *Lacticaseibacillus rhamnosus*, entrapped in alginate was coated with locust bean gum and xantham gum (Cheow et al., 2014). The presence of Locust gum makes it more resistant to acidic environment and not only protects it from the low pH and prevents the release of probiotic into the simulated gastric juice. This results in the dissolution of the capsule, thereby releasing the probiotic in the simulated intestinal juices. Overall the beads coated with locust bean gum showed a better release profile than the beads coated with xantham gum (Cheow et al., 2014).

The entrapped cells multiply when the microspheres are suspended in an appropriate medium. When the cells reach the critical concentration, which is the volume of the core's volume, the capsules rupture thereby releasing all the cells (Călinoiu et al., 2019). This period is defined as the burst-release time. An experiment that was conducted using *L. casei* entrapped in an alginate matrix in which the burst-release method was practiced, showed that the encapsulation of bacteria can help to create a significant lag in time that protects the cells from harsh conditions of the external environment during production, storage and consumption

(Basu et al., 2018). At the same time, the delay in releasing is not too long which ensures the release of the microbe in the target site.

The encapsulation of probiotics should not affect its antimicrobial activity negatively so that its efficiency is not affected. A study was conducted in which *Pediococcus acidilactici*, *Limosilactobacillus reuteri*, and *L. salivarius* was coated with inulin and alginate. The results showed a considerable reduction in the antimicrobial activity by the encapsulated cells than the microbial activity by the non-encapsulated cells (Atia et al., 2016).

To improve the survival of the probiotics, an antibacterial encapsulating material is needed. Poly glutamic acid films were developed with different concentration of polylysine in an attempt to produce antibacterial packaging material to protect a probiotic bacteria that produces GABA. When less PL was added, the antimicrobial activity was quite ineffective. But as the quantity of PL added to the PG increased, the antimicrobial activity of the material also increased significantly (Karimi et al., 2020).

Many bacteria are sensitive to high temperature and UV rays so encapsulating them would provide protection and increase their viability. In research, *Lactiplantibacillus plantarum* PCM 2675 was encapsulated in sodium alginate by electrospinning (Mahmoud et al., 2020). Both the free bacterial cells and the immobilized cells were subjected to harsh conditions such as UV radiation and high temperature. It was evident that both showed a reduction in metabolic activity after the exposure. But, it must be noted that the inhibition of growth of the immobilized cells was less compared to that of the free cells (Žur et al., 2016).

## Synergistic interaction of bioactive compounds with probiotics

Over the years, probiotics have shown to be efficient, not only ameliorating the gut microbiome but also ameliorating the risks factors of various conditions like obesity, metabolic syndrome, and skin problems. (Li et al., 2021). The synergistic effect is defined as the cumulative effects of two active ingredients when they interact with each other leading to the cumulative effect of their activity. The synergistic effect of the probiotic strain along with bioactive agents has shown a positive impact on the gut by increasing the gut microbiota and reducing the risks of obesity, hypertension, and kidney disease. (Xavier-Santos et al., 2020). These have also proven to be efficient in weight loss in obese and overweight individuals. The interaction of the probiotic strains with bioactive agents has been studied over the years in humans, chickens, dogs, and mice (Wiciński et al., 2020; Xavier-Santos et al., 2020).

## Antioxidants

When probiotics and antioxidants are administered in the appropriate quantities, their synergistic interaction results in reducing the risks of kidney disease. In dogs, the effects supplemented the parameters of blood along with the biochemical profile of the urinary system (Meineri et al., 2021). Probiotics, prebiotics, and antioxidants also showed low inflammation, and improved renal function due to antioxidant supplementation. Thus, the use of probiotics and antioxidants along with prebiotics maintains good nutritional status and improves blood and urinary parameters (Meineri et al., 2021). Few probiotic products also showed antioxidant properties. When fermented milk obtained from mammals like cows, goats, and camels was supplemented with probiotic of the bacterial strain *Pedococcus pentosus*, the antioxidative property was shown in Balakrishnan and Agrawal (2014). This also displayed increased radical scavenging activity (Meineri et al., 2021). It is observed that when hairless mice orally consumed live *BBY*, *BBY* prevented UV-induced trans epidermal water loss in the mice. The hydrogen peroxide level was suppressed and oxidation of proteins, lipids and xanthine was observed (Meineri et al., 2021).

The two strains of bacterial species *Bacillus subtilis* PB6 and *Bacillus cereus* were observed for their antioxidant activity and immune responses in chicken broiler (Abudabos et al., 2016). The use of probiotics showed positive modulation in oxidants and antioxidant levels. An increase in the antioxidant level, reduced glutathione concentration, and inhibited lipid peroxidation was observed (Abudabos et al., 2016). Reduced inflammation and obesity in Type 2 diabetic mice was observed in strains of *S. boulardii*. This particular effect of *Saccharomyces boulardii* resulted in dramatic differences in the gut microbial composition of the host organism. Human clinical trials also showed increased antioxidative activity and anti-atherogenic effects when treated with *L. fermentum* ME-3 from fermented milk. Thus, probiotics as antioxidants have exhibited reduced oxidative damage radicals, scavenging rats. The superoxide dismutase activity was also reduced significantly in the human body (Meineri et al., 2021).

## Antimicrobials

Due to the increase in antibiotic resistance observed in pathogens, antimicrobial agents are introduced to combat the growing infections. The genus *Bacillus* is capable of producing antimicrobial agents which is an elementary alternative to combat antibiotic-resistant pathogen, it has proven to be an efficient alternative to the antibiotics used. *Bacillus* species possess properties such as antibacterial, antifungal, and antitumor. (Caulier et al., 2019). Polypeptides like

bacitracin, along with antimicrobial agents, have successfully treated colitis and diarrhoea caused by *Clostridium difficile*. Cyclic and anionic AMP subtilisin exude antimicrobial activity against agents like bacterial vaginosis and related pathogens. Seaweed probiotics also synergistically improved the survival of shrimp. This seaweed probiotic has an antimicrobial effect against the pathogens hindering the survival of shrimp (Sumi et al., 2015).

Probiotics, with the help of antimicrobial activity, produce antimicrobial substances like bacteriocins, and organic acids, which modulate the host's immune system (Igbafe et al., 2020). Bacteriocin-like compounds have proven to be responsible for the antimicrobial property exhibited by *Bifidobacterium*. This *Bifidobacterium* also inhibits pathogens like *E. coli*, and *L. monocytogenes*. Even Lactic acid bacteria (LAB) and certain yeasts have an antimicrobial effect against many pathogens (Igbafe et al., 2020). Bacterial species like *Bifidobacterium longum*, *L. rhamnosus*, and *L. delbrueckii* have the ability to self-aggregate and allow their antimicrobial activity to interact and aggregate with other microorganism like the wound pathogens *Staphylococcus aureus* and *Candida albicans* (Frassinetti et al., 2020).

## Liquid delivery system or encapsulation through the liquid system

When delivered to the site of action, probiotics may be under several alterations which may weaken their overall effect due to changes in pH, oxygen, temperature, and storage condition. These probiotics show vulnerability towards several environmental factors such as temperature and pH, thus, the maintainance of the viability of probiotics in such stresses by developing successful probiotic delivery systems is important (Govender et al., 2014). Encapsulation protects the probiotic or the bacteria by forming a layer over it that can withstand extreme conditions upon its delivery to the site of action. The materials used in the process of encapsulation are sure that they are non-toxic to the particles to be encapsulated. Encapsulated materials can also be stored for long durations and increased shelf life (Govender et al., 2014). The liquid delivery systems include processes like sol-gel immobilization, extrusion, emulsification, coacervation, and emulsion gel.

## Sol-gel immobilisation

This method encapsulates the bacteria in a three-dimensional framework by an aqueous polymerization or gelation reaction. In this method of encapsulation, bacteria or probiotic are covered with a layer of polymers that are stimuli-responsive soluble-insoluble polymers (Penhasi, 2015). The

biomaterials used for encapsulating also include polysaccharides like alginate, chitosan, and agarose, and proteins like collagen and gelatin. Strains of *Bifidobacteria* are the most commonly used in experiments. Research have shown that encapsulation of these strains ensures protection and longevity (O'Callaghan & Van Sinderen, 2016). Under the survival test using Salt solution and PIF dispersion methods, the viability displayed by encapsulated cells was more than that of the free cells (non-encapsulated cells) (O'Callaghan & van Sinderen, 2016).

*Ligilactobacillus salivarius*, a probiotic strain, was coated using alginate and alginate–gelatin microgels and was subjected to various conditions to check viability. When both the encapsulated and free probiotics cells were introduced to artificial saliva, their viability was maintained. However, it was also noted that after incubation under simulated conditions of the GIT, the survival of encapsulated cells was greatly improved compared to that of the non-encapsulated cells (Kwiecień & Kwiecień, 2018). Further, these cells were stored for five weeks to verify the survival rate. Encapsulated *L. salivarius* cells showed improved viability than the free counterparts. Under thermal stability testing of the encapsulated cell, it was noted that the encapsulated cells displayed higher viability of cells after the heat treatment. This is to mimic the simulated thermal processing used in the food industry (Kwiecień & Kwiecień, 2018).

### Extrusion

In this method, hydrocolloid is mixed with probiotics, which is then introduced in the extruder. This extruder is usually a syringe. The syringe, under pressure drops the contents present inside into a gelling mixture. The gelling mixture is subjected to constant stirring while the contents of the syringe drop into it (Muhardina et al., 2018). The drops formed in the gelling solution are called beads, and the size depends on the diameter of the syringe needle used, and it also depends on the gap between the syringe needle and the gelling solution. The core of the beads is a porous network. This way, the cells are trapped in a 3-dimensional network. *L. bulgaricus* probiotic in capsule was formed after extrusion encapsulation with alginate. These were tested under low temperatures, and it was observed that the encapsulated *L. bulgaricus* showed tolerance to lower temperatures. It can be concluded that this probiotic capsule is in a cold or frozen food product (Muhardina et al., 2018).

### Emulsification

This technique is based on the homogenization of continuous and discontinuous phases. Probiotics are made into a slurry paste or lyophilized powder mixed with hydrocolloids like alginate, carrageenan, and pectin (discontinuous phase)

(Tee et al., 2014). The stirring speed and the ratio of water in oil affect the size and diameter of the droplets formed. These droplets formed are called capsules which have a liquid core. This mixture is then stabilized using emulsifiers. The capsules are recovered by settling and recovered usually have unequal size and shape (Frakolaki et al., 2021).

*Bifidobacterium longum* was encapsulated the using emulsification method. The encapsulated and free cells were tested for survival ability under heat treatments. It was observed that at 65 °C, the cells that were encapsulated had higher viability when compared to the free cells. Similarly, when the survival of cells was checked under long-term storage, encapsulated cells showed resistance to a lower temperature (Ji et al., 2019). *L. plantarum* was encapsulated by the method of emulsification. The viability cells of the strain were noted when it was exposed to pH as low as pH 2.0 and exposed to bile salts. In pH 2.0, a comparison was drawn between the encapsulated and non-encapsulated cells of probiotic strain *L. plantarum*. It is observed that the encapsulated cells were higher in quantity when compared to free cells (Tee et al., 2014).

### Coacervation

In the coacervation method, the core material is first scattered in the hydrophobic bifunctional phase. This core was emulsified in a continuous phase that has two or more different polymers (Bosnea et al., 2014). Bacterial strains *Lactiplantibacillus paraplantarum*, and *L. paracasei* were microencapsulated using coacervation. The viability of encapsulated *L. paraplantarum*, and *L. paracasei*, the non-encapsulated and encapsulated *L. paraplantarum* and *L. paracasei* were verified by subjecting it to low pH of 2.0. The coacervated *L. paraplantarum* and *L. paracasei* showed resistance to the low pH retaining their population with 89% of their initial viability (Bosnea et al., 2014). *L. acidophilus* was encapsulated by complex coacervation, after which was subjected to transglutaminase crosslinking, which yielded improved resistance of the encapsulated material and enhanced protection (Bosnea et al., 2014; Tee et al., 2014). The encapsulated cells showed increased viability when exposed to pepsin to mimic the gastro-intestinal environment. Under heat treatment, i.e., 63 °C for 1800 s and 72 °C for 15 s, the encapsulated cells showed improved resistance due to crosslinking with transglutaminase (Tee et al., 2014).

### Emulsion gels

A 3-dimensional protein network is formed. This network entraps the oil droplets emulsified when gelling of the continuous phase occurs due to the treatment of heat, salt, and acid (Torres et al., 2016). The viability of *B. breve* cells that were encapsulated had notably increased when a comparison

was drawn with the viability of non-encapsulated *B. breve* cells (Kwiecień & Kwiecień, 2018). Different strains of probiotic bacteria, like *B. longum*, and, *L. plantarum* were encapsulated using the emulsion method by materials like calcium alginate beads to verify the heat parameter. They were exposed to heat (65 °C). After an incubation period of 30 min, the bacteria that were encapsulated displayed increased survival potential than the non-encapsulated cells (Kwiecień & Kwiecień, 2018).

### Solid delivery system or encapsulation through drying methodology

For the probiotics to be delivered under extreme stresses like heat, pH, and enzyme degradation these probiotics need to be encapsulated. This encapsulation will ensure the viability of the probiotics as well as protection from degradation by various factors (Govender et al., 2014). The various solid delivery systems include spray drying, electrospray drying, electrospinning, and spray chilling (Govender et al., 2014). Figure 3 shows the preparation of encapsulated probiotics and their types.

#### Spray drying

Spray drying is a conventional method to encapsulate heat-sensitive material like probiotics, and flavor. When *L. reuteri* was directly spray-dried using whey from the slurry fermentation, it was tested for survival rate under simulated intestinal conditions. It was observed that the encapsulated cell count of *L. reuteri* had increased upon introduction to the intestinal juice because the encapsulated bacteria were released while increasing the survival rate to 54% after 3 h (Jantzen et al., 2013). After incubation with digestive juices, free cells of *L. reuteri* showed loss of 86% in live cells, while the encapsulated bacteria showed a loss of only 54% under similar parameters. It was noted that the survivability of *L. reuteri* was increased when whey matrix was used for encapsulation by 32% against free/non-encapsulated ones (Jantzen et al., 2013).

#### Electrospray drying

The probiotics were first introduced into a capillary. This method relies on the application of electric fields, leading to deflection in the particles with variants. The electrospraying by deposition in solution method (Gomez-Mascaraque et al., 2016) has three variants of the electrospraying technique, electrospraying in solution technique, coaxial electrospraying technique, and electrospraying deposition technique (Tapia-Hernández et al., 2017). When *L. plantarum* was encapsulated using electrospray drying, it was proved

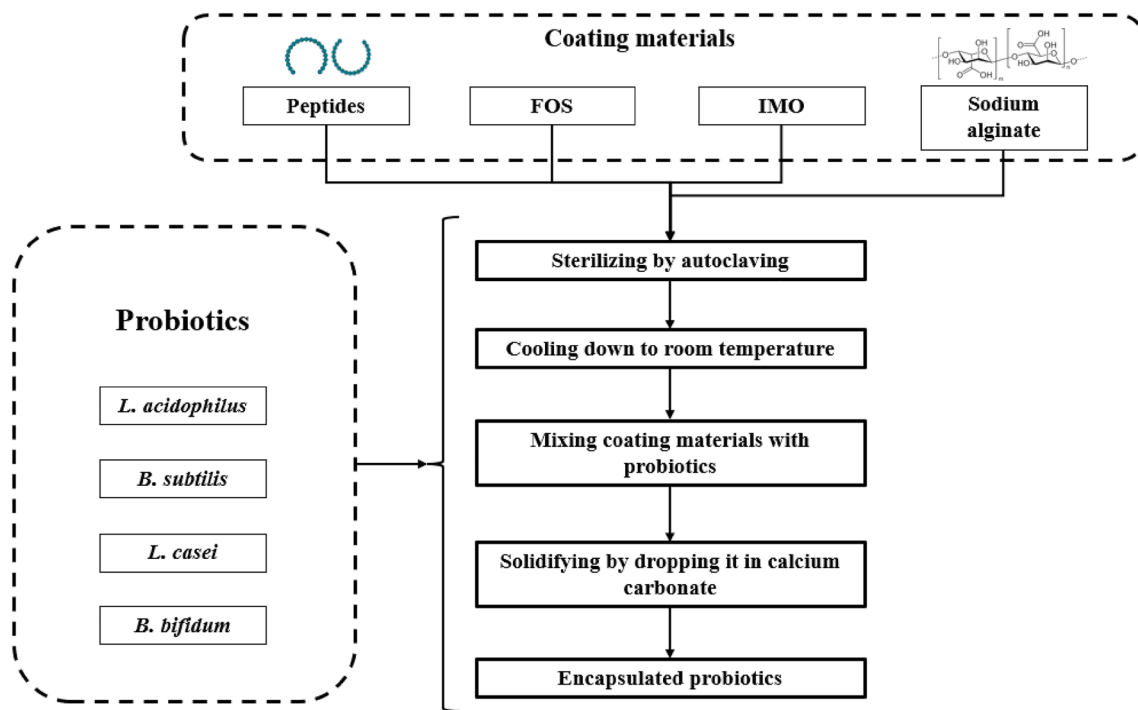
efficient and reliable as 96% of *L. plantarum* culture notably retained upon the application of electric field and osmotic stresses (Tapia-Hernández et al., 2017).

#### Electrospinning

Electrospinning involves the continuous production of nanofibers for encapsulation of the probiotic. This method involves the liquid droplet getting electrified in order to generate a jet. An electrospinning setup comprises a spinneret, a high-voltage supply, a syringe pump, and a collector (Mojaveri et al., 2020). Utilizing chitosan/alginate of multi-layer fibre mats to encapsulate *Bacillus coagulans*, it was found that it protects these probiotic cells against gastrointestinal and improves its attachment, followed by growth in the intestine. Multiple layers of coating may ensure efficient protection of the incorporated probiotics (Anselmo et al., 2016). Electrospun bacteria have been in the form of polymers and have found applications in biomedical research. *Staphylococcus epidermidis* was introduced to carboxymethyl cellulose/polyethylene oxide fibres to treat diabetic foot ulcers. Bacterial strain *Lactobacillus* was loaded into PVA and polyvinyl pyrrolidone fibres to successfully treat bacterial vaginitis (Deng & Zhang, 2020). Electrospun probiotics show promising results as they display controlled delivery and active packaging. Encapsulated probiotics can replenish naturally occurring microorganisms that are destroyed when the body is invaded by diseases like psoriasis. These have also found use in tissue engineering. Even burns and scars have shown healing to some extent. Regeneration of skin tissues and protection from UV rays was also observed (Deng & Zhang, 2020). A summary of encapsulation techniques with their advantages and disadvantages are represented in Table 3.

In spite of the relative advantages of electrospinning blends for encapsulating probiotics, there are some limitations, like the lack of protection for active ingredients or controlled release mechanisms. Combining coaxial electrospinning with biomaterials has overcome these limitations in the delivery of probiotics. Plant cells contain cellulose, which is a polysaccharide that is good for strength and stiffness in nanofibers. Combining carboxymethyl cellulose with PEO has been shown to be effective for encapsulating *S. epidermidis* (Kurečič et al., 2018).

The addition of protective agents to nanofibers can greatly enhance the vitality of probiotics. In a study, (Škrlec et al., 2019), *Lactobacillus plantarum* was encapsulated in polymer materials with and without lyoprotectants. The protectants not just did not interfere with said electrospinning preparation procedure, but they significantly prevented bacterial survivability loss during the production and storage periods (Kurečič et al., 2018). They reported that probiotic quantities and lyoprotectant classes of the polymer



**Fig. 3** Preparation of encapsulated probiotics. *FOS* Fructooligosaccharide, *IMO* Isomaltose-oligosaccharide

**Table 3** Merits and demerits of techniques employed in encapsulation—a sum-up

Encapsulation techniques	Advantages	Disadvantages
In situ polymerization	Inexpensive wall materials and simple manufacturing equipment	Highly complex procedure. An important precursor, formaldehyde, has a pungent smell and high toxicity for higher animal life
Interfacial polymerization	Simple process and lower emphasis on monomer purity	Time-consuming; high cost of polymer monomer
Spray drying	Simple procedure	High energy consumption
Solvent evaporation	Simple procedure	Low drug-loading efficiency
Emulsion-based Processes	Precise control of droplet size can be achieved,	
Hot-Melt Extrusion (HME)	Microparticles filling into gelatin capsules perfectly	thermoplastic excipients can be intimately mixed. material at the surface can be released too quickly, be degraded, or impart unpleasant taste, and release is not constant over time
Core-shell Microcapsules	Taste-masking, chemical protection of the active, or control over release kinetics	High pH needed
Coacervation	Simple procedure	Chemically hard
Concentric Nozzles	Yield monodisperse core-shell particles	Drying droplets is difficult
Liposomes method	Prevent vitamin C losses Provided some stability to vitamin C	Stability lost above 121 °C
Micro encapsulation with PGMS (polyglycerol monostearate)	Highest efficiency	Iron release was 12% when stored at 4 °C for 3 days
Ultrasonic dispersion method	Higher encapsulation efficiency	Slower in vitro release

were significantly linked to probiotic viability. Furthermore, Trehalose outperformed sucrose in regards to storage protection. In complement to lyoprotectants, probiotics were

useful as an additional ingredient in electrospinning. FOS is a saccharide that can help lactobacillus flourish. It may be utilized as a cladding for prophylactic encapsulation using



**Table 4** A comparative overlook of all encapsulation techniques

	In situ polym- erization	Interfacial polymerization	Spray drying	Solvent evaporation	Emulsion- based processes
Stability	Stable	Stable	Stable	Stable	Stable
Preparation	Simple	Difficult	Simple	Simple	Difficult
Cost	Low	High	Low	High	High
Binding force	Strong	Strong	Strong	Strong	Strong
Application	Selective	Selective	Wide	Wide	Selective
Microbial protection	No	No	No	No	No

electrospinning technology, which not only does not harm bacteria but also maintains vitality and improves thermal stability (Feng et al., 2018; Škrlec et al., 2019). Various parameters of all the encapsulation techniques related to its preparation were elaborated in Table 4.

### Future perspective and summary

A wide array of food products containing probiotic strains are available in markets today, consumption of which in a prescribed amount is found to provide numerous health benefits. Various new technologies are used for the isolation and production of probiotics. Bioengineering of probiotics using new fermentation technologies using suspended and immobilized cell technologies are found to be effective. Several studies indicate favorable results when using different encapsulation procedures and encapsulation material. Although probiotic encapsulation technology has advanced significantly, further research is still needed to make this technology commercially more successful. There are still many challenges that needs to be overcome, such as selecting non-toxic encapsulation material, better encapsulation technology, more pH resistant polymers as coating materials and cost management. Further, we need to have a complete understanding of the physiochemical characteristics of the coating materials in order to predict or to have a control over the release of the probiotics under different environmental conditions such as pH, temperature, salinity. Cost of the product depends upon the polymer used for encapsulation, amount of product produced, and the encapsulation technique used. Moreover, the cost of the probiotic encapsulation technology has to be minimized.

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### Declarations

**Conflict of interest** All authors declares that they have no conflict of interest to disclose.

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