***Review Article***

**A Comprehensive Review of Preservation Techniques for Probiotic Lactic Acid Bacteria: Focus on Cryopreservation and Lyophilization**

**Abstract**

Lactic acid bacteria (LAB) are crucial for food fermentation and offer nutritional and health benefits, particularly as probiotics. However, maintaining their viability and functionality during processing and storage remains a significant challenge. This comprehensive review examines the effectiveness, benefits, and limitations of cryopreservation and lyophilization techniques for preserving probiotic LAB strains. Cryopreservation involves storing biological materials at ultra-low temperatures to prolong viability, whereas lyophilization removes water through sublimation, enabling room temperature storage and easier transportation. The survival of LAB during these processes is influenced by factors such as the bacterial strain, cultivation conditions, cryoprotectants, and processing parameters. Cryoprotectants such as trehalose and lactose provide additional protection against stress-induced damage. Despite these advancements, further research is needed to develop strain-specific preservation protocols, enhance probiotic survivability in the gastrointestinal tract, and improve cost efficiency for large-scale applications. Challenges include differences between strains, maintenance of probiotic stability and functionality over time, and making these methods practical and cost-effective for industrial use. By identifying research gaps, this review aims to guide future studies to optimize LAB preservation techniques and ensure their sustained efficacy for health benefits and industrial utilization. Addressing these issues is essential for the successful incorporation of probiotics into functional foods and their widespread application in promoting human health.

**Key words:** Lactic acid bacteria, Probiotics, Cryopreservation, Lyophilization, Viability, Functionality, Preservation techniques

1. **Introduction**

**Lactic acid bacteria (LAB)** represent a diverse group of industrially significant microorganisms extensively employed in the fermentation of various food products, including dairy, bakery, meat, legume, and beverage items (Abedin et al., 2024). LAB contribute significantly to the enhancement of food quality by improving nutritional content and sensory attributes such as flavor and texture. Moreover, they offer documented health-promoting effects, particularly supporting gastrointestinal health and overall physiological well-being (Meena et al., 2025). The dominant species include *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Weissella,* and *Bifidobacterium* (Jaffar et al., 2023; Meena et al., 2022). These gram-positive, non-spore-forming bacteria thrive in low-pH environments and are facultative or obligate anaerobes. LAB conduct homo- or heterofermentative metabolism, producing lactic acid and compounds such as ethanol, CO₂, and diacetyl . They are extensively used in industrial food fermentation processes and are recognized for their safety under the designation "Generally Recognized As Safe" (GRAS) (Edalatian Dovom et al., 2023).

Nutrition is increasingly recognized as a tool for promoting health, with functional foods, particularly probiotics, gaining popularity (Abedin et al., 2024). Probiotics are live microorganisms that provide health benefits when consumed in sufficient amounts (Ji et al., 2023). Probiotics help to reshape the balance of gut microorganisms in humans and inhibit the growth of harmful bacteria in the intestine (You et al., 2022). The benefits of probiotics are primarily associated with their ability to modulate the gut microbiota, reduce nutritional intolerance (such as lactose intolerance), enhance the bioavailability of macro- and micronutrients, and mitigate allergic reactions in sensitive individuals (Meena et al., 2023). For a food product to qualify as a probiotic, it must contain at least 106 colony-forming unit (CFU) of probiotic microorganisms per gram. To achieve health benefits, daily intake of 107–109 CFU is recommended for human consumption (Gul & Durante-Mangoni, 2024). Currently, numerous microorganisms are recognized as bacterial probiotics, with a significant proportion belonging to lactic acid bacteria (LAB), particularly *Lactobacillus*, *Leuconostoc*, and *Streptococcus*. Additionally, several non-LAB microorganisms, such as specific species of *Bifidobacteria*, *Bacillus*, and *Escherichia coli*, are widely acknowledged as probiotics (Vera-Santander et al., 2023). To be widely accepted, probiotics must be cost-effective, stable during processing and storage, and maintain a minimum viability of 6–7 log CFU/mL at ingestion (Singh et al., 2022).A crucial step in the formulation and production of probiotic products is ensuring the viability of beneficial bacteria throughout technological processes and extended storage periods (Sulabo et al., 2020). LAB frequently encounter stresses, such as extreme temperatures, acidic environments, osmotic pressure, and nutrient deprivation, which can lead to bacterial death and negatively impact fermentation and metabolite production (Gao et al., 2022; Meena et al., 2024).

Since ancient times, humans have relied on ice and snow to preserve various food products. Although refrigeration and freezing have long been used for food preservation, they are widely employed to maintain the overall food quality (Bebartta et al., 2024a). Freeze-drying is the most widely used method for preserving LAB, as it has been demonstrated to achieve higher survival rates than most other drying techniques (Gagneten et al., 2024). Freeze-dried lactic acid bacteria (LAB) are utilized as starter cultures in fermented foods and are incorporated into dry foods, pharmaceuticals, and biomaterials (Atanasov et al., 2023). Freeze-drying or lyophilization is a widely used technique for converting liquid LAB cultures into stable dry powder forms. However, technical challenges, such as abrupt temperature fluctuations during the process, can lead to increased cell mortality and a significant reduction in LAB viability . The extreme conditions encountered during freezing, drying, and post-processing critically affect bacterial cell viability. These stresses induce mechanical and solute-related damage, compromise the integrity of the cell wall and DNA, lead to protein denaturation and functional inactivation, and cause chemical and osmotic disruptions, resulting in a substantial reduction in cell viability (Atanasov et al., 2023). To reduce stress and enhance stability during freeze-drying and subsequent storage, lyoprotective media was added to the bacterial cultures. These lyoprotectants consist primarily of carbohydrates, with trehalose and lactose being the most extensively studied components (Cui et al., 2022). These methods extend the shelf life and address global resource scarcity by effectively minimizing waste.

Cryoprotectants help to maintain microbial viability by preventing ice recrystallization, protecting the cell membrane, and offering antioxidant activity (Meena et al., 2023). Although cryopreservation and lyophilization are widely used to preserve probiotic lactic acid bacteria, important gaps remain in the literature. There is limited research on optimizing these methods for different probiotic strains, as most studies have applied general protocols without considering strain-specific needs. Additionally, the effects of these preservation techniques on the functionality of probiotics, such as their ability to survive in the gut and to provide health benefits, are not fully understood. Challenges related to maintaining probiotic viability and stability during long-term storage require more attention. Furthermore, there is a lack of studies on making these preservation methods more cost-effective and practical for large-scale industrial applications. This review summarizes the current knowledge on preserving probiotic lactic acid bacteria using cryopreservation and lyophilization. It examines the effectiveness, benefits, and limitations of these methods. The review also highlights challenges, such as differences between strains, keeping probiotics stable and functional over time, and making these methods practical and cost-effective for large-scale use. By identifying gaps in research, this review seeks to guide future studies to improve these preservation techniques and ensure that probiotics remain effective in health and industrial applications.

**2. Cryopreservation**

Cryopreservation refers to the storage of biological materials at low temperatures in order to prolong their viability. Historical evidence shows that since approximately 2000 BC, icehouses in Mesopotamia have been used to preserve food by maintaining cold conditions (Bojic et al., 2021). At cryopreservation temperatures, chemical and biological reactions in living cells slow down significantly or cease entirely, enabling long-term preservation of various biological samples (Chang & Zhao, 2021). Cryopreservation is regarded as one of the most effective strategies to extend the shelf life of food products. However, the formation and recrystallization of ice during freezing can cause substantial structural damage to cells and tissues, leading to quality deterioration such as weight loss, discoloration, protein denaturation, and nutrient depletion (Jaiswal & Vagga, 2022). Extreme conditions during freezing, drying, and post-processing can severely damage bacterial cells and affect their viability. Such damage includes mechanical and osmotic stress, disruption of the cell wall and DNA, protein denaturation, and chemical injury, all of which reduce cell survival (Chen et al., 2022). Particularly, in cell-based frozen foods like freeze-dried probiotics, ice crystal-induced mechanical stress can significantly reduce cell viability, to mitigate these adverse effects, the incorporation of antifreeze agents during cryogenic processing or storage is essential to protect food structure and maintain product quality

**2.1 Principles and mechanisms of freezing and thawing**

The freeze-thaw process during cryopreservation induces significant chemical and physical stress, which constitutes the primary mechanism responsible for the degradation of cryopreserved biological samples (Chang & Zhao, 2021). Exposure of cells to sub-zero temperatures in the absence of cryoprotectants is typically lethal. Given that water constitutes approximately 80% of the tissue mass, the freezing of intracellular and extracellular water exerts a predominant influence on the biochemical and structural alterations associated with unprotected freezing-induced injury (Whaley et al., 2021). The cryopreservation protocol includes preparation, controlled freezing, thawing, and post-thaw recovery. There are two main theories explaining the damaging effects of freezing on cells: (1) ice crystals physically damage cell membranes, preventing cells from remaining structurally intact after thawing, and (2) a harmful rise in solute concentration occurs in the remaining liquid as ice forms inside the cells during cooling (Whaley et al., 2021). During preparation, cells are suspended in a cryoprotectant solution, with the choice of cryoprotectant and its concentration optimized based on cell type and application. Controlled freezing employs a gradual cooling rate, typically 1 °C/min, to mitigate ice crystal formation, with samples cooled to −80 °C before storage at −196 °C in liquid nitrogen vapor. Thawing was conducted rapidly in a 37 °C water bath to minimize cryo-injury, followed by washing to eliminate residual cryoprotectants that may exhibit cytotoxicity at high concentrations. Post-thaw recovery involves culturing cells in suitable growth media, with viability evaluated via Trypan Blue exclusion or flow cytometry (Aarattuthodi et al., 2025).

**2.2 Mechanics of cryopreservation**

Cryopreservation involves rapid freezing, ultra-low-temperature storage, thawing, and rehydration to maintain food quality (Bebartta et al., 2024a). In the first stage, rapid freezing (~1°C/min) is performed using blast or liquid nitrogen freezers to prevent the formation of large ice crystals, which negatively impacts the texture and structure. Food is cooled below its glass transition temperature, where it becomes rigid, thus minimizing ice expansion (Liu et al., 2021). After freezing, the food is stored at ultralow temperatures (−80°C to −196°C) in airtight containers to avoid freezer burns caused by ice sublimation, which dehydrates and damages the food. During thawing, slow warming prevents structural damage owing to rapid ice expansion. Rehydration restores moisture, flavor, and texture to their original state (Bebartta et al., 2024a). Cryoprotective agents (CPAs), such as glycerol, dimethyl sulfoxide (DMSO), and propylene glycol, are added to lower the freezing point of water and protect cells. Optimal CPA concentrations (5–15%) reduce salt crystal formation and mechanical damage. CPAs, typically of low molecular weight (<100 Da), penetrate effectively, retain water, and exhibit low toxicity, making them widely used in food preservation and pharmaceuticals (Sharma & Sharma, 2022).

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**Figure 1**: Cryopreservation involves either slow cooling, which allows controlled water efflux to prevent intracellular ice, or vitrification, which uses rapid freezing and/or high cryoprotectant levels to prevent ice formation. Ice growth is controlled by nucleation and recrystallization inhibitors. Devitrification can occur if warming is too slow, causing ice formation. Apoptosis inhibitors help reduce cell death from cryopreservation-induced stress (Bojic et al., 2021)

**2.3 Types of cryopreservation**

Cryopreservation processes can generally be classified into distinct types: slow freezing, vitrification (where the aqueous environment of the cell or tissue solidifies into a non-crystalline, glass-like phase), non-freezing sub-zero preservation, and dry state preservation (Jaiswal & Vagga, 2022). The main objective is to maintain viability and functionality throughout the freezing and thawing processes. The choice of method typically depends on the type of biospecimen and its specific preservation requirements (Aarattuthodi et al., 2025).

**2.3.1 Vitrification**

Cryopreservation techniques, including slow freezing and vitrification, are primarily differentiated by the concentration of cryoprotective agents (CPAs) and applied cooling rates (Jaiswal & Vagga, 2022). Vitrification is a physical phenomenon distinct from traditional freezing that occurs at the glass transition temperature, typically between −80 and −130 °C. During vitrification, the sample transitions into a solid amorphous state without the formation of ice crystals, thereby preserving structural integrity at the molecular level (Bojic et al., 2021). Vitrification, which is characterized by a rapid cooling rate, prevents intracellular ice formation (IIF) by instantly creating a glass-like structure. Although it demands the use of highly concentrated, toxic cryoprotective agents (CPAs) and advanced expertise, it has recently gained interest because of its ability to entirely eliminate ice crystal formation (Ishizaki et al., 2023). However, excessive CPA exposure can induce osmotic imbalance and cytotoxicity, resulting in cytoskeletal disruption, spindle apparatus destabilization, and chromosomal de-condensation (X. Wang et al., 2023).The primary limitation of vitrification is the occurrence of ice nucleation and devitrification during the warming phase, which can cause severe damage to cryopreserved samples (Chang & Zhao, 2021). Cryoprotective vitrification uses cryoprotectants to increase cell viscosity and prevent ice nucleation at cryogenic temperatures (Sharma & Sharma, 2022). Vitrification is more complex than slow freezing because of the need for precise regulation of cooling and warming rates as well as the requirement for specialized equipment. Moreover, not all cell types and tissues are compatible with vitrification, necessitating the development of tailored protocols for different biological materials (Aarattuthodi et al., 2025).

**2.3.2 Slow freezing**

Slow freezing involves a gradual cooling process that minimizes intracellular ice formation by dehydrating cells. This method is commonly applied to individual dispersed cells, as it is effective even with low levels of toxic cryoprotective agents (CPAs) and requires only basic operator proficiency (Ishizaki et al., 2023). Slow freezing typically occurs at a rate of 1°C/min using a portable freezing container or controlled-rate freezer. It uses low CPA concentrations (<2M) to reduce the contamination risk and the need for advanced skills. However, it can cause cryo-injury due to extracellular ice formation (Kuang et al., 2022). Despite the use of controlled cooling, intracellular ice crystal formation or cellular dehydration may still occur, compromising the post-thaw viability. Furthermore, the procedure is inherently time intensive, which presents a significant challenge when processing large sample volumes (Aarattuthodi et al., 2025).

**3. Role of cryoprotectants**

Cryopreservation encounters significant challenges, including high costs and maintenance demands of specialized equipment, freeze burns leading to textural degradation, and the risk of psychrophilic microbial contamination during freezing and thawing, which collectively affect preservation efficacy and food quality (Bebartta et al., 2024a). Thus, the use of antifreeze agents is essential in frozen food applications to mitigate mechanical damage caused by ice crystal formation during cryogenic processing or cryopreservation (Chen et al., 2022). Cryoprotectants are used only to preserve bacteria when they are stored in a frozen state (Bodzen et al., 2021).

* Cryoprotectants are widely utilized to protect cells during freeze-drying, reducing damage caused by ice crystallization, membrane rupture, and osmotic stress.(Nguyen et al., 2020).
* CPAs are compounds used to minimize the damage caused by freezing during cryopreservation.
* It should dissolve easily at low temperatures and in high concentrations.(Bojic et al., 2021)
* They should exhibit low toxicity.
* CPAs need to be able to penetrate cells.
* They must be biologically compatible or acceptable (Jaiswal & Vagga, 2022).
* Cryoprotectants (CPAs) are often utilized to enhance cell survival rates by reducing damage to cells during cryopreservation (X. Wang et al., 2023)

**3.1 Types of cryoprotectants**

A variety of chemical compounds with high solubility have been utilized to safeguard biological cells against cold-induced damage, thereby markedly enhancing the post-thaw viability. These compounds are termed cryoprotective agents (CPAs) or cryoprotectants (Sharma & Sharma, 2022). The selection of an appropriate cryoprotectant is crucial because it plays a key role in preserving the structural integrity of the cell membrane and enhancing overall cell viability during and after cryopreservation (Nguyen et al., 2023). Commercially available CPAs are primarily categorized as permeating or non-permeating, based on their ability to enter cells (Chang & Zhao, 2021) (Figure 2).

1. Cell-permeating agents rapidly penetrate cells and prevent the formation of intracellular ice crystals, although their level of toxicity to cells (Kuang et al., 2022). These include dimethyl sulfoxide (DMSO), glycerol, and 1,2-propanediol (Jaiswal & Vagga, 2022).
2. Conversely, non-permeable cryoprotectants, such as polymers, polyvinyl pyrrolidone, hydroxyethyl starch, 2-methyl-2,4-pentanediol, and various saccharides, function externally to protect cells (Jaiswal & Vagga, 2022).

Furthermore, any cryoprotectant considered for use must be non-toxic and biocompatible to ensure safety and compatibility with biological systems (Nguyen et al., 2020).

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**Figure 2**: Comparative illustration of penetrating and non-penetrating cryoprotectants across cell membranes

**Table 1**: The brief classification of cryoprotective agents

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| **Permeable cryoprotectant agents**  **(Small molecules)** | **Non-permeable cryoprotectant agents** | |
| Dimethyl sulphoxide | Sugars | Polymers |
| Ethylene glycol | Sucrose | Polyethylene glycol |
| Propylene glycol | Trehalose | Polyvinyl pyrrolidone |
| Glycerol | Raffinose | Hydroxy ethyl starch |
| Methanol | Mannitol | Ficoll |
| Ethanol | Glucose | Serum proteins (mixture) |
| Glycine betaine | Galactose | Milk proteins (mixture) |

**Table 2:** The different types of cryoprotectants, along with their specific functions, are summarized in the table below.

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| **Cryoprotective agent** | **Effects and features** | **Reference** |
| Glycerol | Their small size allows them to penetrate cell membranes, protecting cells by interacting with water molecules. | (Jungare et al., 2022) |
| Skim milk | Skim milk, rich in lactose and proteins, preserves bacteria better due to higher water retention in proteins compared to sucrose. | (Bodzen et al., 2021) |
| Trehalose | It is a glucose dimer linked by an α-1,1-glycosidic bond, with an acetal linkage that enhances stability under extreme temperatures and resistance to acid hydrolysis at low pH. | (Whaley et al., 2021) |
| Sucrose | * Widely used to protect lactic acid bacteria. * Creates an amorphous structure. * Binds to membrane proteins through hydrogen bonds. * Causes water to flow out of cells initially. | (Bodzen et al., 2021) |
| DMSO (Dimethyl sulfoxide) | * It reduces the electrolyte concentration in the unfrozen fluid surrounding cells at any temperature. * It is cost-effective and exhibits relatively low cytotoxicity. | (Jaiswal & Vagga, 2022) |
| Ethylene glycol | It alters the hydrogen bonding when mixed with water and, water starts making the same amorphous solid. | (Jungare et al., 2022) |
| Propylene glycol | Minimizes ice crystal formation and supports cell stability during cryopreservation, functioning similarly to ethylene glycol. | (Aarattuthodi et al., 2025) |

1. **Lyophilization (Freeze Drying)**

Lyophilization, commonly known as freeze-drying, is an extensively validated and effective technique for preserving bacterial viability during the drying process. It is currently regarded as the most efficient method for dehydrating bacteria while maintaining viability (Meena et al., 2023). Freeze-drying is a crucial and effective preservation technique for maintaining the long-term stability of bioactive products (Girardeau et al., 2022). Freeze-drying is a widely employed method for preparing dried starter cultures for food applications (Ibrahim et al., 2023). Lyophilization removes intracellular water from bacterial cells, reducing the water activity (aw) to ≤0.2. This significant decrease in aw suppresses or completely inhibits cellular metabolic processes (Bodzen et al., 2021).

**4.1 Principle of lyophilization**

The freeze-drying process consisted of three main stages: freezing, sublimation (primary drying phase), and secondary drying. Sublimation plays a crucial role in the lyophilization process by enabling the direct transition of water from its solid (ice) state to vapor, bypassing the liquid phase (Sourabh Bhosale et al., 2021) . During the freezing stage (critical for maintaining bacterial viability), ice first forms in the extracellular medium because of the higher solute concentration inside the cells than in the surrounding medium. This extracellular ice formation increases the solute concentration outside the cells, creating an osmotic gradient between intracellular and extracellular environments (Bodzen et al., 2021). The removal of water during lyophilization is driven by the concentration gradient of water vapor between the drying front and condenser. Sublimation occurs under specific conditions, namely, at pressures around 4.58 mm Hg and temperatures below the triple point of water (approximately 0.0098 °C). At the triple point, the solid, liquid, and gaseous phases of water coexist at equilibrium at the same temperature and pressure (Sourabh Bhosale et al., 2021).

**4.2 Mechanism of lyophilization of lactic acid bacteria**

Freeze-drying and lyophilization have become the reference processes for LAB preservation lactic acid bacteria. This process removes most of the water at temperatures well below 0°C, typically between −40°C and −20°C, through sublimation, which is the transition from ice to water vapor (Fonseca et al., 2021a). During freezing, LAB cells are subjected to cold and osmotic stresses, causing their lipid membranes to become more rigid, the cells to lose water, and their volume to shrink (Girardeau et al., 2022). These stresses can cause significant cellular damage, resulting in a loss of viability and a reduction in functional activities such as acid production, aroma compound generation, and probiotic functions.(Fonseca et al., 2021b). The survival of microorganisms throughout this process is influenced by several factors, such as the intrinsic resistance properties of the bacterial strains, initial microbial concentration, growth conditions, drying medium, presence of protective agents, storage conditions (temperature, atmosphere, and relative humidity), and rehydration procedures (Ibrahim et al., 2023). Various strategies have been proposed to enhance bacterial resistance to stress.

1. The incorporation of protective agents prior to stabilization.
2. the optimization of processing parameters such as freezing rate, product temperature during freeze-drying, residual moisture content, and storage conditions; and
3. physiological adaptation of bacterial cells during fermentation (Fonseca et al., 2021b).

Freezing is suitable for a broad range of sample volumes, whereas vitrification is typically limited to smaller applications (Bojic et al., 2021). Freeze-dried samples can be stored for up to five years at 4 °C or room temperature, ideally with a desiccant, such as silica gel, to avoid rehydration. Alternatively, they can be stored at -80 °C (Corrales et al., 2023). This dry cryopreservation method facilitates the storage and transport of biomaterials, while minimizing reliance on extensive equipment (Aarattuthodi et al., 2025).

**Table 3:** Factors influencing freeze drying

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| **Factors** | **Impact** | **Reference** |
| Sample size | Small size yield more uniform lyophilized product | (Arora et al., 2024) |
| Surface area and thickness of the sample | Uniform drying in large surface area | (Bodzen et al., 2021) |
| Sample characteristics | Physico-chemical traits (structure, texture) of product | (Kuang et al., 2022) |
| Condenser temperature | Affect the water vapour removal from product; temperature and characteristics of product | (Arora et al., 2024) |
| Freezing (FZ) rate | Size of ice crystal formed and its distribution pattern | (Corrales et al., 2023) |
| Number of cycles | Overall production efficiency | (Gagneten et al., 2024) |
| Chamber pressure | Moisture content on lyophilized product; product integrity and its stability | (Arora et al., 2024) |
| Environmental factor | Quality, stability and shelf life of product | (Fonseca et al., 2021b) |

1. **Review of preservation methods: Cryopreservation and lyophilization**

Cryopreservation and lyophilization are key methods for preserving bacterial cultures, each with distinct advantages. Cryopreservation, involving ultra-low temperatures (-80°C to -196°C), ensures high viability and minimal metabolic changes, making it ideal for long-term storage, but it requires energy-intensive equipment and cryoprotectants (Sharma & Sharma, 2022). In contrast, lyophilization, or freeze-drying, offers portability, ease of storage at ambient temperatures, and long shelf life, though it can lead to viability loss due to stresses during the process (Arora et al., 2024). The choice between these methods depends on factors like storage duration, cost, and portability needs. By understanding how each process works, like cooling and thawing in cryopreservation or freezing and drying in freeze-drying, we can improve existing methods and possibly create entirely new ways to preserve materials more effectively. Therefore, it is essential to develop preservation methods that are both cost-effective and efficient. Freeze-drying holds great promise in this regard, as it can significantly lower the cost of storing samples, particularly in developing countries. However, further research is crucial to optimize this method for reliable and consistent storage and recovery (Jungare et al., 2022).

1. **Application of freeze drying to preserve LAB**

Freeze-drying, or lyophilization, is a widely accepted method for the long-term preservation of lactic acid bacteria (LAB), offering the advantage of maintaining viability and functionality during storage. However, the survival of LAB during freeze-drying is influenced by various factors, including the bacterial strain, cultivation conditions, type and concentration of cryoprotective agents, and processing parameters. Although freeze-drying can achieve high survival rates, it often induces cellular stress due to freezing and dehydration, potentially leading to membrane damage, protein denaturation, and metabolic impairment. Therefore, optimizing the freeze-drying protocol with suitable cryoprotectants is essential to minimize viability loss and ensure effective preservation of LAB strains.

**Table 4:** Various applications of freeze-drying LAB using different cryoprotectant agents

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| **Applications** | **Details** | **LAB Strain** | **References** |
| Investigated diverse cryoprotective strategies to enhance LAB survival | Reported high freeze-drying stability and long-term viability (4–8 months) across several LAB strains | *L. fermentum* N2 and TC 3-11, *Weissella confusa* NN1, *L. rhamnosus NA1-8*, and *L. paracasei* ***AV2-1*** | (Atanasov et al., 2023 |
| Beneficial impact of sorbitol-enriched microencapsulation systems | Survival rates were:   * 67.1%-free cells * 89.4%-cryoprotected microencapsulated cells * 91.2% - sorbitol-enriched microcapsules.   Viability remained significantly higher (p < 0.05). | *Lactobacillus plantarum* | (Jouki et al. 2021) |
| Use of functional plant-based additives as cryoprotectants | Sodium alginate combined with pumpkin powder (natural coating) provided enhanced protection not only during freeze-drying but also under simulated gastrointestinal conditions, highlighting its potential for improving probiotic delivery | *L. plantarum MG989*, *L. fermentum MG901*, *L. lactis MG5125*, *S. thermophilus MG5142*, and *E. faecium MG89-2* | (Nguyen et al., 2020) |
| Effects of different cryoprotectants on LAB cells | After freeze drying for one year:   * S5 (skim milk + 5% sodium glutamate) -100% recovery and 87% viability * S4 (skim milk) - 96% recovery, 74% viability * S3 (trehalose) - 7% viability   S2 (sucrose) was the least effective protectant., | *Lactobacillus fermentum* | (Stefanello et al., 2019) |
| Addition of 1% (m/v) L-theanine as a cryoprotectant during freeze-drying | Probiotics coated with theanine showed higher viability after freeze-drying than the uncoated strains. However, for some probiotics, there is no notable improvement in the adhesion rate, survival in the gastrointestinal tract, or results from accelerated stability test | *Lactiplantibacillus plantarum* MG5023, *Enterococcus faecium* MG5232, *Lactococcus lactis* MG4668, *Streptococcus thermophilus* MG5140, and *Bifidobacterium animalis ssp. lactis* MG741 | (Kwon, 2023) |

1. **Comparative evaluation between cryopreservation and lyophilization for lab storage**

Cryopreservation and lyophilization both techniques aim to maintain cell viability and preserve bacterial characteristics during storage. Cryopreservation offers high survival rates and genetic stability but requires specialized equipment and continuous maintenance of low temperatures. Lyophilization, on the other hand, allows for room temperature storage and easier transportation of cultures, but may result in lower viability rates and potential changes in bacterial properties. The choice between these methods depends on factors such as the specific LAB strain, intended storage duration, available resources, and downstream applications. Researchers must carefully consider these aspects when selecting the most appropriate preservation technique for their LAB strains.

**Table 5** : Comparative analysis of cryopreservation and lyophilization for Lactic Acid Bacteria (LAB) storage

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| Aspects | Cryopreservation | Lyophilization |
| Definition | Freezing LAB cells at ultra-low temperatures (Bebartta et al., 2024a) | Freeze-drying LAB cells by sublimation of water under vacuum (Fonseca et al., 2021a). |
| Temperature range | −80°C to −196°C (Aarattuthodi et al., 2025) | Ambient to -20°C for post-drying storage |
| Moisture content | High, cells stored in aqueous medium (Aarattuthodi et al., 2025). | Very low, typically <2% moisture content (Bodzen et al., 2021). |
| Storage stability | Long-term stability if consistently stored at ultra-low temperatures (Bebartta et al., 2024). | Long-term stability at ambient or refrigerated conditions (Corrales et al., 2023). |
| Cell viability loss | Moderate during freezing and thawing due to ice crystal damage (Jungare et al., 2022). | Higher during drying if lyoprotectants are inadequate (Gao et al., 2022). |
| Impact on lab activity | Minimal loss if freezing and thawing are controlled (Whaley et al., 2021) | Potential activity loss due to drying-induced stress (Ibrahim et al., 2023). |
| Additives required | Cryoprotectants (e.g., glycerol, DMSO) to prevent ice damage (Jaiswal & Vagga, 2022) | Lyoprotectants (e.g., trehalose, sucrose) to protect during drying (Whaley et al., 2021). |
| Metabolic activity post- preservation | Retained but suppressed at low temperatures (Noufeu et al., 2025) | May decrease if drying or rehydration is suboptimal (Bodzen et al., 2021) |
| Ease of handling | Requires specialized freezers or liquid nitrogen systems (Bajerski et al., 2021) | Easier handling and transport due to lightweight dried form (J. Wang et al., 2025) |
| Cost implications | High operational cost due to energy-intensive equipment (Jungare et al., 2022). | Moderate initial cost but lower operational/storage expenses (Jungare et al., 2022). |
| Transportation | Challenging; requires cold chain logistics (Jungare et al., 2022). | Easy; dried LAB are stable without refrigeration (Aarattuthodi et al., 2025). |
| Environmental impact | Higher energy consumption due to freezing needs (Gao et al., 2022) | Lower energy demand; more sustainable (J. Wang et al., 2025) |
| Example of LAB strains | Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus plantarum. (Jouki et al., 2021) | Lactobacillus rhamnosus, Lactococcus lactis. (Atanasov et al., 2023) |
| Suitability for LAB | Ideal for sensitive LAB strains requiring high viability retention (Ishizaki et al., 2023) | Best for applications needing room-temperature stability and portability (Atanasov et al., 2023) |

1. **Preservation challenges for LAB**

Preservation of lactic acid bacteria (LAB) poses several challenges due to their delicate nature and specific environmental requirements. During the production and application of starter cultures, LAB often experience challenging conditions such as temperature shifts, acidic environments, osmotic pressure, and limited nutrient availability (Chen et al., 2022). In cell-based frozen foods, such as freeze-dried probiotics, the formation of ice crystals can cause mechanical damage that greatly reduces cell survival (Bebartta et al., 2024b). Freeze-drying, particularly during the freezing stage, can cause significant damage to bacterial cells, leading to their death upon rehydration . This emphasizes the importance of carefully managed storage conditions. Factors such as water activity (aw), temperature, light exposure, and the gaseous environment play a crucial role in maintaining bacterial viability. However, even under controlled conditions, probiotic preparations commonly experience a reduction in viability, often exceeding 1 log unit over a month of storage at 25°C. The mechanisms of cellular damage are varied and include protein aggregation, lipid oxidation, and the Maillard reaction involving reducing sugars (Bodzen et al., 2021). Most commercial probiotic strains today are highly sensitive to temperature, as their optimal viability is adapted to the human body temperature. Exposure to extreme temperatures—either too low or too high—can severely affect their survival. Low temperatures increase membrane porosity, causing leakage of intracellular contents, while high temperatures can inactivate essential molecular machinery like polymerases (Singh et al., 2022). During freeze-drying, the drop in temperature subjects bacterial cells to cold stress, while the formation of ice crystals creates osmotic stress due to the increased concentration of solutes. Additionally, the desorption stage introduces mechanical stress, as the removal of water involves breaking hydrogen bonds within the cells (Gagneten et al., 2024). High osmotic pressure can slow their growth, lower their survival rates, and reduce their metabolic activity (Chen et al., 2022). Thus, to minimize cell death during freeze-drying and storage, commonly employed strategies include regulating the cooling rate, incorporating lyoprotectants, and maintaining optimal storage conditions.

1. **Conclusion and future prospects**

Lactic acid bacteria (LAB) play a pivotal role in food fermentation by offering both nutritional and health benefits, particularly as probiotics. However, maintaining cell viability and functionality during processing and storage remains a significant challenge. Cryopreservation and freeze-drying are effective preservation methods, and cryoprotectants such as trehalose and lactose provide additional protection against stress-induced damage. Despite these advancements, further research is needed to develop strain-specific preservation protocols, enhance probiotic survivability in the gastrointestinal tract, and improve the cost efficiency for large-scale applications. Addressing these gaps will enable the optimization of LAB preservation techniques, ensuring their sustained efficacy for health benefits and industrial utilization. Cell recovery after freezing is a major challenge during cryopreservation. In some instances, ice crystals can form within cell samples, leading to cellular damage and the potential failure of some cells to recover. To mitigate this, many laboratory and clinical protocols employ cryoprotective agents to protect cells from ice crystal formation. Additionally, they regulate freezing and thawing rates to prevent temperature shock that could harm cells. While lyophilization offers many benefits, it also presents challenges, including the complexity of the process, the need for sterile vehicles for reconstitution, higher costs, maintenance of the desired moisture content in the final product, and difficulties in scaling from lab to production. Overcoming these hurdles is essential to ensure successful lyophilization.

Future perspectives for preserving probiotic lactic acid bacteria, particularly through cryopreservation and lyophilization, encompass several promising avenues. Optimization of protective agents tailored to specific probiotic strains is crucial for enhancing survival rates and maintaining functionality. Integration of nanotechnology, such as nanoencapsulation methods, could improve bacterial stability during preservation and storage. Exploring synergistic effects by combining cryopreservation and lyophilization with other preservation methods may yield enhanced results. Genetic engineering of probiotic strains to increase resistance to preservation-induced stress could potentially improve survival rates and maintain probiotic properties. Implementation of advanced analytical tools and omics technologies will provide deeper insights into the molecular mechanisms of bacterial survival during preservation. Standardization of industry-wide protocols for cryopreservation and lyophilization is essential to ensure consistent quality and viability of probiotic products. Research into sustainable and eco-friendly preservation techniques will reduce environmental impact and improve cost-effectiveness. Exploring preservation methods suitable for personalized probiotic formulations and investigating novel storage conditions and packaging materials to extend shelf-life are also important areas of focus. Lastly, developing rapid and accurate quality control methods will ensure the continued efficacy of preserved probiotic products.

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