***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. Overcoming these challenges is essential for the effective incorporation of probiotics into functional foods and their broader use in promoting human health.

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

**1. Introduction**

Lactic acid bacteria (LAB) are a diverse group of microorganisms widely utilized in the fermentation of various food products—including dairy, bakery, meat, legume, and beverages—due to their significant industrial and nutritional value (Abedin et al., 2024). According to Codex Alimentarius Standard CXS 243-2003, fermented milks like yogurt must contain at least 10⁷ CFU/g of viable microbes at the end of their shelf life, while non-fermented products require a minimum of 10⁶ CFU/g (Mukherjee et al., 2022). LAB improve food quality by enhancing both nutritional content and sensory attributes such as flavor and texture. They also confer well-documented health benefits, especially in supporting gastrointestinal health and overall physiological function (Meena et al., 2025). Prominent LAB genera 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 exhibit either facultative or obligate anaerobic metabolism. Through homo- or heterofermentative pathways, they produce lactic acid along with by-products like ethanol, CO₂, and diacetyl. Owing to their established safety profile, LAB are generally recognized as safe (GRAS) and are extensively used in industrial food fermentation (Edalatian Dovom et al., 2023). As functional foods gain prominence, probiotics—live microorganisms that offer health benefits when consumed in adequate amounts—are increasingly in demand (Ji et al., 2023; Abedin et al., 2024). These microbes help balance gut flora, inhibit pathogenic bacteria, alleviate lactose intolerance, improve nutrient absorption, and modulate allergic responses (Meena et al., 2023; You et al., 2022). For a product to qualify as probiotic, it must contain at least 10⁶ CFU/g, with a recommended daily intake of 10⁷–10⁹ CFU to ensure effectiveness (Gul & Durante-Mangoni, 2024). While LAB such as Lactobacillus, Leuconostoc, and Streptococcus are prominent probiotic strains, other non-LAB microbes, including Bifidobacteria, Bacillus, and Escherichia coli, are also widely recognized (Vera-Santander et al., 2023). For probiotics to be viable in commercial products, they must remain stable during processing and storage and retain at least 6–7 log CFU/mL viability at the point of consumption (Singh et al., 2022). Ensuring bacterial viability throughout processing and shelf life is therefore a critical step in probiotic product development (Sulabo et al., 2020). LAB are frequently subjected to environmental stresses—such as extreme temperatures, acidic pH, osmotic pressure, and nutrient scarcity—which can reduce viability and impair fermentation efficiency (Gao et al., 2022; Meena et al., 2024). In response, global efforts, including initiatives by the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU44), are working to establish standardized international guidelines for probiotic products. These underscore the need for validated preservation techniques backed by robust scientific evidence. Traditional preservation methods like refrigeration remain in use, but advanced technologies such as freeze-drying (lyophilization) have become the gold standard for preserving probiotic LAB (Bebartta et al., 2024a). Freeze-drying converts liquid cultures into shelf-stable powders with higher survival rates than conventional drying methods (Gagneten et al., 2024). Freeze-dried LAB are commonly used as starter cultures in food and are increasingly incorporated into pharmaceuticals, nutraceuticals, and biomedical products (Atanasov et al., 2023). However, the lyophilization process poses several technical challenges. Drastic temperature shifts during freezing, drying, and rehydration can damage cellular structures—causing DNA fragmentation, protein denaturation, and oxidative stress—leading to reduced viability (Atanasov et al., 2023). To combat these effects, lyoprotective agents like trehalose and lactose are often added to stabilize cell membranes and enhance survival (Cui et al., 2022). Similarly, cryoprotectants prevent ice crystallization, preserve membrane integrity, and reduce oxidative damage during low-temperature storage (Meena et al., 2023). Despite their industrial utility, both cryopreservation and lyophilization face persistent challenges. Chief among them is the lack of strain-specific protocols. Most preservation methods are generalized and do not account for the distinct physiological and molecular responses of different strains, such as Lacticaseibacillus casei and L. paracasei (Peralta et al., 2023; X. Wang et al., 2023). As a result, viability and functional performance can vary widely between species. Moreover, the impact of preservation on probiotic functions—such as gut survivability, epithelial adhesion, and immunomodulatory effects—remains underexplored (Guan et al., 2025; Zhang et al., 2022). Additionally, challenges around long-term storage stability, especially under suboptimal conditions, remain unresolved. Oxidative stress, moisture, and food matrix interactions can gradually diminish cell viability, emphasizing the need for more robust stabilization strategies (Musakhanian et al., 2022). Furthermore, few studies have comprehensively evaluated the economic feasibility and scalability of these preservation methods for industrial applications (Kiepś et al., 2023). Taken together, these gaps highlight the need for an interdisciplinary approach to probiotic preservation—one that integrates strain-level physiological profiling, post-preservation functional validation, and techno-economic analysis. This review critically examines current strategies for preserving probiotic LAB, with a focus on cryopreservation and lyophilization due to their industrial relevance. It evaluates their effectiveness, limitations, and the key factors affecting bacterial viability, including strain variability, protective agents, and processing parameters. The analysis draws on peer-reviewed studies from 2017 to 2025 selected for their relevance to probiotic LAB preservation. By identifying unresolved issues such as long-term stability, strain-specific responses, and cost-effective scale-up, this review aims to guide future research toward optimizing preservation techniques that sustain probiotic functionality for both health and industrial applications.

**2. Cryopreservation**

Cryopreservation involves storing biological materials at low temperatures to extend their viability. Historical records indicate that as early as 2000 BC, icehouses in Mesopotamia were used to preserve food through cold storage (Bojic et al., 2021). At cryogenic temperatures, chemical and biological activities within cells slow dramatically or cease altogether, allowing for the long-term preservation of various biological samples (Chang & Zhao, 2021). This makes cryopreservation one of the most effective strategies for prolonging the shelf life of food products. However, freezing presents significant challenges. The formation and recrystallization of ice crystals during freezing can cause structural damage to cells and tissues, leading to quality issues such as weight loss, discoloration, protein denaturation, and nutrient degradation (Jaiswal & Vagga, 2022). In particular, bacterial cells—such as those found in freeze-dried probiotic formulations—are vulnerable to mechanical and osmotic stress, cell wall and DNA disruption, protein denaturation, and other chemical injuries during freezing, drying, and subsequent processing stages (Chen et al., 2022). Ice crystal-induced mechanical stress is a major contributor to reduced cell viability in frozen, cell-based food products. To counter these effects, antifreeze agents are often incorporated during cryogenic processing and storage. These agents help protect cellular structures, reduce mechanical damage, and maintain overall product integrity and quality (Bebartta et al., 2024).

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

Cryopreservation involves a freeze-thaw cycle that imposes significant chemical and physical stress on biological samples, often leading to degradation (Chang & Zhao, 2021). In the absence of cryoprotectants, exposure to sub-zero temperatures is typically lethal to cells. Since water makes up roughly 80% of tissue mass, the freezing of intracellular and extracellular water plays a central role in the biochemical and structural damage seen during unprotected freezing (Whaley et al., 2021). The cryopreservation process includes four key stages: preparation, controlled freezing, thawing, and post-thaw recovery. Two primary mechanisms explain the freezing-induced damage: (1) ice crystal formation physically disrupts cell membranes, compromising structural integrity, and (2) as ice forms, the remaining liquid becomes increasingly concentrated with solutes, which can be harmful to cells (Whaley et al., 2021). During preparation, cells are suspended in a cryoprotectant solution, with the type and concentration tailored to the specific cell type and application. Controlled freezing typically uses a cooling rate of 1 °C/min to reduce ice crystal formation, lowering the temperature to −80 °C before long-term storage in liquid nitrogen vapor at −196 °C. Thawing is performed rapidly in a 37 °C water bath to minimize cryo-injury, followed by washing to remove residual cryoprotectants that may be cytotoxic at high concentrations. Post-thaw recovery involves culturing cells in appropriate media, with viability assessed using Trypan Blue exclusion or flow cytometry (Aarattuthodi et al., 2025).

**2.2 Mechanics of cryopreservation**

Cryopreservation is a preservation method that employs rapid freezing, ultra-low-temperature storage, controlled thawing, and rehydration to maintain the structural and sensory qualities of food products (Bebartta et al., 2024a). During the initial stage, a cooling rate of approximately 1 °C per minute—achieved using blast freezers or liquid nitrogen—prevents large ice crystal formation, thus protecting texture and microstructure. The product is cooled below its glass transition temperature, becoming rigid and minimizing cellular injury from ice expansion (Liu et al., 2021). Samples are then stored at −80 °C to −196 °C in airtight containers to prevent ice sublimation and freezer burn. Controlled, gradual thawing reduces the risk of structural damage from ice recrystallization, while rehydration restores moisture, flavor, and texture (Bebartta et al., 2024a). Cryoprotective agents (CPAs)—such as glycerol, DMSO, and propylene glycol—are added to reduce ice and salt crystal formation, lower the freezing point of water, and preserve cellular integrity. Effective concentrations typically range from 5–15%. These low molecular weight (<100 Da) compounds penetrate cells, retain intracellular water, and have low cytotoxicity, making them suitable for food and pharmaceutical use (Sharma & Sharma, 2022). Two primary cryopreservation strategies exist: slow cooling, which allows controlled water efflux and reduces intracellular ice, and vitrification, which uses ultra-rapid freezing and/or high CPA levels to entirely prevent ice formation. Ice behavior is further controlled using nucleation and recrystallization inhibitors. However, overly slow warming may lead to devitrification—ice formation during thawing—that can compromise cell viability. The addition of apoptosis inhibitors is also being explored to reduce cell death associated with cryo-stress (Bojic et al., 2021), as illustrated in Figure 1.

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**Figure 1**: Schematic representation of cryopreservation mechanisms

**2.3 Types of cryopreservation**

Cryopreservation methods can be broadly categorized into four types: slow freezing, vitrification (in which the cellular environment solidifies into a glass-like, non-crystalline phase), non-freezing sub-zero preservation, and dry-state preservation (Jaiswal & Vagga, 2022). The primary goal across all approaches is to preserve the viability and functionality of biological samples during freezing and thawing. The choice of technique depends on the type of biospecimen and its specific preservation requirements (Aarattuthodi et al., 2025).

**2.3.1 Vitrification**

Cryopreservation techniques such as slow freezing and vitrification differ primarily in cooling rates and the concentrations of cryoprotective agents (CPAs) employed (Jaiswal & Vagga, 2022). Vitrification is a rapid cooling process in which biological samples transition into a solid, glass-like (amorphous) state at cryogenic temperatures—typically between −80 and −130 °C—without the formation of ice crystals (Bojic et al., 2021). This process effectively prevents intracellular ice formation (IIF), which can otherwise disrupt cellular membranes and organelles. **Although vitrification requires highly concentrated and potentially toxic CPAs as well as specialized expertise, it has gained growing interest due to its ability to completely eliminate ice crystal formation (Ishizaki et al., 2023).** However, Ishizaki et al.’s findings were based on model systems under controlled laboratory conditions, which may not fully reflect the complexity of industrial-scale probiotic preservation. Excessive CPA exposure may cause osmotic imbalance and cytotoxic effects, leading to cytoskeletal disruption, spindle apparatus destabilization, and chromosomal decondensation (X. Wang et al., 2023). While Wang et al. provided detailed insights into cellular damage mechanisms, their study primarily focused on mammalian cells rather than bacterial systems, limiting direct applicability to LAB. A major limitation of vitrification is **devitrification**, which occurs during the warming phase and involves the recrystallization of the amorphous structure into damaging ice crystals (Chang & Zhao, 2021). However, Chang and Zhao did not evaluate the influence of variable warming rates on different probiotic strains, leaving gaps in strain-specific vulnerability data. Cryoprotective vitrification mitigates this risk by increasing intracellular viscosity to suppress ice nucleation at low temperatures (Sharma & Sharma, 2022), although most supporting studies emphasize theoretical mechanisms or simulations rather than empirical validation in LAB. Despite its advantages, vitrification remains technically demanding due to the need for precise control of both cooling and warming rates, as well as the use of specialized equipment. Additionally, not all cell types or probiotic strains respond uniformly to vitrification, necessitating the development of strain-specific protocols (Aarattuthodi et al., 2025). However, Aarattuthodi et al. did not assess the long-term viability or functional activity of LAB post-vitrification, limiting conclusions about real-world effectiveness.

While systematic reviews specific to vitrification of Lactic Acid Bacteria are still limited, broader analyses on LAB viability under freezing and refrigerated conditions offer valuable comparative insights. A recent systematic review and meta‑analysis covering 278 studies on lactic acid bacteria in refrigerated and frozen food matrices found significant variability in viability depending on preservation method, formulation, and adjunct components. **Initial microbial load, food matrix structure, genus (**e.g., Lactobacillus vs. Bifidobacterium), and the use of **prebiotics** emerged as key determinants of survival trends (Siddiqui et al., 2024). These findings support the interpretation that, although vitrification has theoretical advantages in preventing intracellular ice formation, **real-world viability outcomes depend critically on strain-specific factors, protective formulations, and application context rather than the preservation method alone.**

**2.3.2 Slow freezing**

Slow freezing is a gradual cooling technique that reduces intracellular ice formation by promoting cellular dehydration. It is widely used for individual dispersed cells due to its simplicity, low toxicity requirements, and minimal need for technical expertise (Ishizaki et al., 2023). Typically conducted at a rate of 1 °C/min using either portable freezing containers or controlled-rate freezers, this method relies on low concentrations of cryoprotective agents (CPAs) (<2 M), which helps minimize cytotoxicity, contamination risk, and handling complexity. While effective for mammalian cells, the applicability of slow freezing to probiotic bacteria—especially under diverse industrial or environmental conditions—remains insufficiently explored. Ishizaki et al.’s work, for example, focused exclusively on mammalian cell lines. Similarly, although (Kuang et al., 2022) detailed the mechanisms of ice-induced damage, their study reported only short-term viability data and did not evaluate post-thaw functionality in lactic acid bacteria (LAB). Despite its advantages, slow freezing can still cause cryo-injury due to extracellular ice formation. Even under controlled conditions, residual intracellular ice or excessive dehydration may compromise membrane integrity, reducing probiotic viability after thawing. Moreover, the method is inherently time-intensive, making it less suitable for high-throughput or industrial-scale applications. Aarattuthodi et al. (2025) acknowledged these limitations but did not examine scalability or economic feasibility—factors that are essential for commercial adoption.

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**3. Role of cryoprotectants**

Cryopreservation faces several challenges, including high costs, the need for specialized equipment, freeze burns that degrade texture, and the risk of psychrophilic microbial contamination during freezing and thawing. These factors collectively compromise preservation efficiency and food quality (Bebartta et al., 2024a). To mitigate mechanical damage caused by ice crystal formation during cryogenic processing, antifreeze agents are widely employed in frozen food applications (Chen et al., 2022). In microbial preservation, cryoprotectants (CPAs) are critical for maintaining bacterial viability during frozen storage (Bodzen et al., 2021). CPAs are also essential during freeze-drying, where they protect cells from ice crystallization, membrane rupture, and osmotic stress (Nguyen et al., 2020; X. Wang et al., 2023). For effective protection, ideal CPAs should:

* Dissolve readily at low temperatures and high concentrations (Bojic et al., 2021)
* Exhibit low cytotoxicity
* Penetrate cell membranes efficiently
* Be biologically compatible or acceptable (Jaiswal & Vagga, 2022)

By fulfilling these criteria, CPAs significantly enhance cell survival rates during both cryopreservation and lyophilization.

**3.1 Types of cryoprotectants**

A wide range of highly soluble chemical compounds, known as cryoprotective agents (CPAs), are used to protect biological cells from cold-induced damage, significantly improving post-thaw viability (Sharma & Sharma, 2022). Selecting an appropriate CPA is critical, as it helps preserve membrane integrity and enhances overall cell survival during and after cryopreservation (Nguyen et al., 2023). CPAs are broadly classified as permeating or non-permeating based on their ability to enter cells (Chang & Zhao, 2021) (Figure 2).

1. **Permeating CPAs**, such as dimethyl sulfoxide (DMSO), glycerol, and 1,2-propanediol, diffuse across cell membranes and inhibit intracellular ice crystal formation, though they may exhibit varying degrees of cytotoxicity (Kuang et al., 2022; Jaiswal & Vagga, 2022).
2. **Non-permeating CPAs**, including polymers (e.g., polyvinyl pyrrolidone, hydroxyethyl starch), 2-methyl-2,4-pentanediol, and saccharides, act extracellularly to stabilize cells by osmotically balancing water movement and preventing external ice damage (Jaiswal & Vagga, 2022).

For effective application, cryoprotectants must be both non-toxic and biocompatible (Nguyen et al., 2020). While formal meta-analyses on CPAs for lactic acid bacteria (LAB) are limited, comparative studies highlight the benefits of specific agents. For instance, (Orhan et al.,2024) found that among ectoine, trehalose, and sucrose, ectoine offered the highest protection for Streptococcus thermophilus cultures frozen at −80 °C. Similarly, factorial experiments combining glucose, sucrose, skim milk, and glycine have shown enhanced viability, stress tolerance, and metabolic activity, underscoring the superior performance of multi-component formulations over single agents. Collectively, these findings emphasize that while the distinction between permeating and non-permeating CPAs is important, optimal protection often requires tailored, multi-component strategies—particularly for LAB preservation under industrial conditions.

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**Figure 2**: Types of cryoprotectants based on membrane permeability

**Table 1**: Classification of cryoprotective agents into permeable (small molecules) and non-permeable types.

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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:** Comparative features of cryoprotective agents used in microbial preservation.

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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., 2023a). 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).

* 1. **Principle of lyophilization**

The freeze-drying (lyophilization) process comprises three sequential stages: freezing, primary drying (sublimation), and secondary drying. Sublimation is a pivotal step, facilitating the direct transition of ice to vapor without passing through the liquid phase, thereby minimizing structural damage to biological materials (Sourabh Bhosale et al., 2021). During the initial freezing stage—critical for preserving bacterial viability—ice nucleation typically occurs in the extracellular medium due to osmotic imbalances, as intracellular solute concentrations are higher than those in the surrounding environment. This results in an osmotic gradient that drives water efflux from the cells (Bodzen et al., 2021). The efficiency of water removal during lyophilization is governed by the vapor pressure gradient between the drying front and the condenser. Sublimation is achieved under low-pressure conditions (approximately 4.58 mm Hg) and temperatures below water’s triple point (~0.0098 °C), where all three phases of water coexist in thermodynamic equilibrium (Sourabh Bhosale et al., 2021). Empirical studies underscore the importance of integrating cryoprotectants—such as skim milk, trehalose, and lactose—into lyophilization protocols to enhance post-process viability. For instance, Jofré et al. (2023) reported ≥94% survival of probiotic cultures following lyophilization, with a viability reduction of ≤0.9 log CFU over 39 weeks of storage; comparable results were observed by Ibrahim et al. (2023b). These findings emphasize that, while adherence to the physicochemical principles of lyophilization is essential, the ultimate preservation efficacy is highly dependent on the selection of compatible strains and tailored lyoprotective formulations.

* 1. **Mechanism of lyophilization of lactic acid bacteria**

Lyophilization, or freeze-drying, is widely regarded as the gold standard for preserving lactic acid bacteria (LAB). This technique removes water at sub-zero temperatures (typically between −40 °C and −20 °C) through sublimation—the direct transition of ice to vapor—thereby minimizing thermal damage (Fonseca et al., 2021a). During freezing, LAB cells are exposed to cold and osmotic stress, leading to membrane rigidification, water efflux, and cellular shrinkage (Girardeau et al., 2022). These stresses can cause significant structural and functional damage, resulting in reduced viability and impaired metabolic activities, including acid production, aroma generation, and probiotic efficacy (Fonseca et al., 2021b). Microbial survival during lyophilization is influenced by multiple factors, including strain-specific resistance, initial cell concentration, cultivation conditions, composition of the drying matrix, type and concentration of protective agents, storage conditions (temperature, humidity, and atmosphere), and rehydration protocols (Ibrahim et al., 2023a). To enhance resistance and viability, several strategies have been proposed:

(i) incorporation of protective agents prior to drying,  
(ii) optimization of processing parameters such as freezing rate, product temperature, residual moisture, and storage stability, and  
(iii) physiological adaptation of bacterial cells during fermentation (Fonseca et al., 2021b).

Compared to vitrification—which is generally restricted to small volumes—freeze-drying accommodates a broader range of sample sizes (Bojic et al., 2021). Lyophilized samples can be stably stored for up to five years at 4 °C or ambient temperature, ideally with desiccants (e.g., silica gel) to prevent moisture uptake, or at −80 °C for long-term preservation (Corrales et al., 2023). This dry cryopreservation approach enables convenient storage and transport of probiotic cultures while minimizing reliance on specialized cold-chain infrastructure (Aarattuthodi et al., 2025).

**Table 3:** Key factors influencing the efficiency and quality of the freeze-drying process.

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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. **Freeze-drying as a method for preserving lactic acid bacteria**

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: Applications of freeze-drying for the preservation of LAB using different cryoprotectants**.

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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 of Cryopreservation and Lyophilization for the Storage of Lactic Acid Bacteria (LAB)**

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., 2023a). |
| 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.

**9. Future Perspectives in the Preservation of Probiotic LAB**

Despite significant progress in the preservation of lactic acid bacteria (LAB), several key challenges remain unaddressed. Future research should focus on **strain-specific preservation protocols**, as the current "one-size-fits-all" approaches often fail to optimize viability and functionality across diverse probiotic strains. Additionally, **novel cryo- and lyoprotectants** derived from natural or GRAS-approved sources should be investigated to improve survival rates without compromising safety. There is a growing need for **integrated preservation systems** that combine process optimization (e.g., controlled freezing rates, pressure modulation) with **omics-based strain profiling** to tailor conditions for each probiotic species. Finally, **scalability and cost-efficiency** in industrial settings, along with compliance with regulatory guidelines (EFSA, FDA, Codex), must be prioritized to ensure real-world applicability of advanced preservation methods.

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**Figure 3:**  Future directions in LAB preservation: A conceptual overview of research priorities.

**10. 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.

Disclaimer (Artificial intelligence)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models.

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