Stabilizing Probiotics by Drying: A Review on Processes, Protective Strategies, and Viability Assessment

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Review

Stabilizing Probiotics by Drying: A Review on Processes, Protective Strategies, and Viability Assessment

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Abstract

Scientific interest in probiotics continues to grow as accumulating evidence links microbiome modulation to improvements in host health. Probiotics are usually described as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host. However, products are exposed to multiple stresses during manufacturing, storage, and gastrointestinal transit. Among these, drying, often employed to stabilize powders and extend shelf life, can impose high viability loss. This review synthesizes recent advances in drying process engineering, formulation design, and viability assessment aimed at improving survival during drying. Freeze drying remains the most widely used technology, while alternative approaches, including conventional spray drying, vacuum drying, spray freeze drying, and electrostatic spray drying, are increasingly evaluated. Protective strategies are discussed, encompassing sublethal conditioning (stress adaptation), optimization of operating parameters, incorporation of excipients, and encapsulation. Lastly, methods for viability assessment are also compared, contrasting culture-dependent assays (e.g., plate enumeration) with culture-independent techniques such as flow cytometry and PCR-based approaches. However, across studies, performance is highly strain-specific, and optimization often entails trade-offs among immediate survival, cycle time, powder stability, and downstream functionality.

Keywords: probiotic; stabilization; drying technologies; process optimization; protective strategies; viability assessment

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1. Introduction

The human body hosts a vast and complex community of microorganisms, collectively known as the human microbiome. This microbial ecosystem plays a pivotal role in modulating host physiology, metabolism, and overall health. Alterations in this microbial ecosystem, a condition known as dysbiosis, have been associated with several diseases. The addition of a sufficient number of beneficial bacteria, or probiotics, has been proposed as a potential strategy to restore microbial balance [1].

Pasteur's discovery relative to the mechanism of fermentation in 1857 marked the beginning of research on the relationship between these microbes and their host [2]. Since then, there have been many definitions of the term probiotic. According to etymology, the word means *in support of life* and comes from the combination of the Greek word *bios* and the Latin preposition *pro*. Early in the 20th century, Metchnikoff was the first researcher to highlight the good effect of probiotics on human health. Lilly and Stillwell coined the term

probiotic in 1965 to refer to "substances secreted by one microorganism which stimulate the growth of another" [3]. Following research developments, later in 1989, Fuller defined these substances as foods containing live microorganisms capable of conferring benefits to the animal host through the improvement of its intestinal microbial balance. The official definition nowadays is the one of the World Health Organisation (WHO) and the Food and Agriculture Organisation of the United Nations (FAO), which has been in effect since 2001. It states that probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host."

A fundamental prerequisite for any microorganism to be designated as probiotic is its demonstrated safety for human consumption and absence of toxicity. Therefore, probiotic strains intended for food or pharmaceutical applications must have a well-established safety profile, evaluated according to their intended use and the applicable regulatory framework. In Europe, the European Food Safety Authority (EFSA) applies the Qualified Presumption of Safety (QPS) approach as a generic, evidence-based safety pre-assessment for selected taxonomic units used in food, provided that relevant strain-specific safety considerations are also addressed. In the United States, microorganisms or microbial-derived ingredients may be considered Generally Recognized as Safe (GRAS) under specific conditions of intended use [4,5]. Probiotic strains and products, as biological preparations, should not only support host health but also be fully characterised and produced in pure or defined combinations. During formulation preparation and subsequent treatments, they should maintain their original characteristics. Indeed, they should be able to withstand harsh processing conditions (heat treatment, acidification, mixing, long term storage, . . .). Furthermore, they must be able to transit and survive in the digestive system while providing a precise dosage form and substance [6]. These microorganisms have strain-specific effects, and the identification of the strain is fundamental for evaluating the effectiveness of the product. Consequently, it is crucial to precisely characterize a probiotic strain, as research findings are strain-specific and cannot be extrapolated to even closely related strains [7].

Probiotics are predominantly composed of lactic acid bacteria (LAB), particularly strains from the genera Lactobacillus and Bifidobacterium, which are also abundantly present in the human intestine. Nonetheless, several strains of Bacilli, Enterococci, Pediococci, Streptococci, and certain yeast, like Saccharomyces cerevisiae, are also recognised as probiotic strains [8]. Lactobacilli and bifidobacteria are generally recognised as safe and commonly added together in commercial probiotic solutions to enhance their benefits [9]. Both Lactobacilli and Bifidobacteria are Gram-positive, non-spore-forming bacteria. However, they differ in oxygen tolerance and morphology: Lactobacilli are non-flagellated rods or coccobacilli that can be either aerotolerant or anaerobic, whereas Bifidobacteria are non-motile bacteria that typically grow under anaerobic conditions with various possible shapes [3]. Beyond established lactic acid bacteria and yeasts, increasing attention is turning to next-generation probiotics, including human-gut-derived strains selected through microbiome-driven approaches such as metagenomics and culturomics. These microorganisms may offer targeted functions, such as barrier reinforcement or immunomodulatory activity, but their translation requires rigorous strain-level safety assessment and fit-for-purpose stabilization and delivery strategies. However, because next-generation probiotics often include strict anaerobes with specific oxygen sensitivity, harvesting constraints, and stabilization requirements, their discussion is limited in the present review. The following sections therefore focus primarily on conventional probiotic microorganisms, particularly lactobacilli and bifidobacteria, for which drying and formulation evidence is more extensively available [10].

Probiotics have attracted significant interest in the field of functional food and pharmaceutical formulations because of their potential health benefits [8]. However, probiotic efficacy is highly strain-, dose-, population-, and indication-specific. However, these effects should not be generalized, as probiotic efficacy is highly strain-, dose-, population-, and indication-specific. Reported benefits include effects on lactose digestion, gastrointestinal function, immune modulation, cholesterol metabolism, and respiratory or otitis-related outcomes, but the strength of evidence varies depending on the specific strain, study design, target population, and clinical endpoint considered [3,11–16]. The biological effects of probiotics may be related to several mechanisms of action, including modulation of host defences through immune and epithelial responses, direct interactions with other microbes through competition or inhibition, modulation of host or microbial metabolites (e.g., bile salts, toxins, short chain fatty acids) and effects of the gut microbiota composition and activity [17,18].

With the rapid expansion of the probiotic market, maintaining microbial viability and functionality during manufacturing and storage has become a critical challenge. Although several reviews have addressed probiotic drying technologies, protective formulations, and viability assessment methods, the available literature remains fragmented. Most studies and reviews discuss individual drying techniques or specific protective strategies separately, whereas direct cross-technology comparisons remain limited. Moreover, the interpretation of probiotic process performance is often complicated by the use of inconsistent viability endpoints, including colony-forming units, flow-cytometry-based counts, membrane integrity indicators, metabolic activity assays, and survival ratios. This review provides an integrated overview of selected literature on probiotic manufacturing, with a particular focus on drying technologies, protective strategies to enhance survival, and methods for assessing viability. By positioning different drying approaches within a common framework, the review highlights current knowledge gaps, discusses the trade-offs between process efficiency, cell survival, and storage stability, and identifies the methodological aspects that currently limit cross-study comparison and industrial translation.

2. Review Methodology

This article was designed as a narrative review aimed at providing a critical and integrated overview of drying technologies, protective strategies, storage stability, and viability assessment methods for probiotic products. The literature search was mainly performed using Google Scholar and Scopus. The final structured search was completed in December 2025, with additional targeted updates included during the peer-review revision process when relevant to address specific topics or recently identified gaps.

The search strategy combined keywords related to probiotics, drying technologies, formulation, stability, and analytical methods. The main search terms included combinations of “probiotic*” with “drying”, “freeze drying”, “lyophilization”, “spray drying”, “spray freeze drying”, “electrostatic spray drying”, “vacuum drying”, “fluidized bed drying”, “encapsulation”, “cryoprotectant”, “lyoprotectant”, “viability”, “survival”, “flow cytometry”, “plate count”, “storage stability”, “water activity”, “glass transition”, “rehydration”, and “functionality”.

Only documents written in English were considered. No strict date restriction was applied, although priority was given to recent publications and to older foundational studies that were considered relevant for the development of the field. Eligible documents included original research articles and review articles. Standards, technical documents, and regulatory documents were also consulted when relevant to terminology, analytical methods, or industrial practice. Studies were included when they addressed at least one of the following aspects: probiotic drying technologies, protective formulations, encapsulation

strategies, survival or viability after drying, storage stability, rehydration behaviour, functionality after processing or storage, or analytical methods for probiotic viability assessment. Studies were excluded when they were not directly related to probiotic or probiotic-related microorganisms, did not address drying, stabilization, storage, formulation, or viability assessment, were focused only on clinical efficacy without technological relevance, or did not provide sufficient methodological information for interpretation.

Titles and abstracts were first screened for relevance, followed by full-text evaluation of selected articles. Reference lists of relevant papers were also checked manually to identify additional sources. Duplicate records were managed using Zotero (Corporation for Digital Scholarship, Vienna, USA) and removed before full-text evaluation. From the selected studies, the main information extracted included probiotic species or strain, drying technology, formulation or protective agents, relevant process conditions, survival or viability outcomes, residual moisture or water activity when available, storage conditions, analytical methods, and the main reported advantages or limitations. Because this is a narrative review, no formal risk-of-bias assessment or quantitative study-quality scoring was performed. However, the evidence was critically interpreted by considering differences in strain, formulation, process conditions, analytical endpoint, and storage conditions, particularly when comparing results across drying technologies or viability assessment methods.

3. Probiotic Production

To be classified as a probiotic, a strain must satisfy stringent functional and safety requirements. This strict set of criteria has driven a rapid expansion of research to identify new strains that can improve both human health and the functionality of food [19]. Strain isolation and identification is the first step when developing a new probiotic product; this is accomplished through a combination of genotypic and phenotypic methodologies. After identification, strains can undergo a series of functional tests first in vitro and then in vivo. Probiotic effects are highly strain-specific, with efficacy also influenced by the surrounding environment and dosage. As a result, it is uncommon for two strains of the same species to have identical probiotic qualities [20].

When developing new strains, great attention is given to assessing their efficiency on a laboratory scale and to optimizing the process conditions required to produce probiotics on a commercial scale. Because each stage of the production process is interdependent, it is crucial to identify strain-dependent phases and implement strategies to maintain cell viability throughout. The transition to commercial-scale production is a critical phase that begins only after a strain has demonstrated robust resilience during the exploratory lab phase. If the strain lacks sufficient resilience, additional research is required to optimize the process [21].

The majority of industrial methods for producing probiotics employ batch fermentation with suspended cells [22]. A fermentation tank is schematically represented in Figure 1.

Upstream fermentation and biomass handling strongly influence the physiological state of probiotic cells and, consequently, their tolerance to dehydration, freezing, heat, oxygen, and osmotic stress. Therefore, fermentation should not be considered only a biomass-production step, but also a conditioning phase that affects subsequent drying performance. The medium typically contains water, a carbohydrate source, nitrogen sources, salts, and micronutrients essential for microbial proliferation. Growth medium composition is a key factor, as it can influence not only biomass yield but also membrane composition, intracellular solute accumulation, acid tolerance, and stress resistance, thereby affecting drying tolerance. Careful development of this mixture is necessary to maximize both biomass

yield and cell robustness [23,24]. Historically, MRS broth (de Man, Rogosa, and Sharpe) has been the most utilized medium for the culture of Lactobacillus and Bifidobacterium species [25]. Seed-train design and inoculation strategy should minimize genetic drift and physiological variability before production-scale fermentation. However, drying tolerance is also strongly affected by the growth phase and fermentation endpoint. The optimal harvest phase is organism-dependent, but most studies focus on the late exponential and stationary phases, with the stationary phase more frequently reported as associated with higher recovery after drying. This improved robustness is often attributed to the activation of stress-response mechanisms, membrane adaptation, and accumulation of protective intracellular compounds. Thus, the fermentation endpoint should be defined not only by biomass yield or cell density, but also by the physiological robustness required for downstream stabilization [23,24]. Fermentation temperature is another critical parameter that must be precisely controlled. Optimal temperature accelerates bacterial proliferation and biomass yield, which is fundamental for attaining high cellular density [19]. In addition to temperature, pH control, agitation, oxygen availability, and fermentation time are key upstream variables. There is general consensus that exposure to certain sublethal adverse conditions during microbial growth can induce tolerance responses that improve resistance to subsequent stresses. For example, changes in pH may affect membrane fatty acid composition and acid tolerance, while temperature shifts can activate heat- or cold-stress responses. Oxygen exposure should also be carefully managed, particularly for oxygen-sensitive strains [23]. Novel approaches to fermentation are also being documented in the literature; these include the addition of cross-flow membranes, fermentation with immobilized cells, and the implementation of continuous processes [22,26].

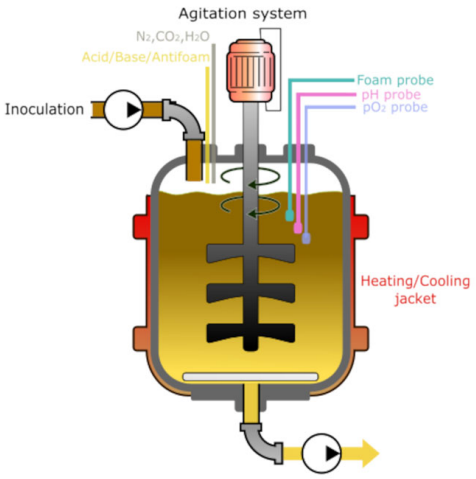

Figure 1. Simplified schematic representation of a stirred-tank bioreactor showing the core components relevant to probiotic biomass production (inoculum line, temperature and gas atmosphere control, agitation, and process monitoring). Original illustration prepared by the authors.

Once fermentation is completed, bacterial biomass is typically separated from the spent medium and concentrated prior to further processing. This step can be achieved through various techniques, including centrifugation, membrane filtration, and other cell-harvesting methods designed to minimize mechanical stress and maintain cell viability. However, harvesting and concentration should also be regarded as critical pre-drying operations, because they can expose cells to shear forces, osmotic shifts, oxygen, temperature changes, and removal of residual broth components that may contribute to protection. Biomass solids concentration and cell density can further affect suspension viscosity, the ratio between cells and protective agents, and drying behavior. The optimal cell concentration is therefore formulation-dependent and should be defined together with the protective medium used,

since different cell-to-protectant ratios can modify both drying kinetics and protection efficiency. In addition, holding time and temperature between harvest, formulation, and drying should be controlled to limit acidification, oxidative stress, metabolic exhaustion, or loss of viability [24].

Industrial manufacturing yields multiple probiotic dosage forms including powders, capsules, and liquid suspensions. Although liquid formulations are the simplest to produce, they generally show low stability and short shelf life [27]. For this reason, final stabilization typically relies on freezing or drying, both of which can reduce cell viability. This loss is mitigated by adding stabilizers (e.g., cryoprotectants for freezing and lyo-protectants for freeze drying) to the formulation. The process must be designed and tightly controlled to achieve the target water activity (a_w), viable cell count, healthy cell population, cell distribution and shelf-life performance. The following sections detail the principal drying technologies and the protective strategies used for probiotic products.

4. Drying Techniques

Liquid cell suspensions are frequently transformed into powdered probiotic preparations to suppress metabolic activity and preserve cell viability during processing, storage, transportation, and consumption. This stabilization is often achieved by removing water content through a drying process, thereby reducing microbial metabolism and generating a stable powdered product with an enhanced shelf life [9]. For long-term powder stability, both residual moisture content and low water activity (a_w) values should be carefully controlled, as they affect molecular mobility, biochemical reaction rates, and microbial viability retention during storage. Water activity describes the availability of free water for chemical and biological reactions, whereas residual moisture content represents the total amount of water remaining in the dried product. However, neither parameter alone is sufficient to predict stability, since intracellular hydration and the glass-transition behavior of the protective matrix also play a critical role. Very low water activity values are often desirable to limit degradative reactions, but the optimal range is formulation- and storage-dependent. However, this dehydration must be precisely controlled to preserve a minimal amount of intracellular water necessary for cell functionality. Excessive dehydration compromises membrane integrity, protein structure, and recovery after rehydration. In such conditions, cells may enter anhydrobiosis, a potentially reversible state of metabolic arrest induced by water limitation. Therefore, the primary challenge in probiotic drying is to inhibit metabolic functions while preventing irreversible cellular damage and maintaining sufficient structural integrity to allow viability retention and recovery during storage and rehydration [19]. Successful stabilization and conversion into powder form yield distinct advantages, including extended shelf life and enhanced cell viability, alongside practical benefits such as simplified handling, reduced storage volume, and broad compatibility for integration into diverse food and nutraceuticals matrices [6].

The preservation of delicate biological structures, such as probiotic cells, constitutes a major challenge in drying research, as these cells are especially vulnerable to thermal, oxidative, and osmotic stresses during the process [28]. Numerous drying processes have been engineered to convert these cultures into powder. The choice among these is governed by multiple parameters, such as the sensitivity of the microbial strain, the physicochemical characteristics of the formulation, the intended application, storage requirements, and economic considerations [9]. Since freeze drying remains one of the most widely used techniques for probiotic stabilization, Table 1 provides a comparative overview of the main drying technologies discussed in this review using freeze drying as the reference process. The table summarizes key practical criteria, including operating temperature, drying time, energy demand, scalability, reported survival, and the main advantages and disadvantages

of each method. The reported survival values should be interpreted as indicative rather than universal, since they refer to representative studies on similar probiotic strains and depend strongly on formulation, process conditions, and analytical methods.

Table 1. Cross-technology comparison of probiotic drying methods using freeze drying as reference technology. Arrows indicate relative trends compared with freeze drying, while survival values are reported from representative studies on similar probiotic strains.

Drying Technique	Operating Temperature	Drying Time	Energy Demand	Scalability	Survival *	Advantages	Disadvantages	References
Spray Drying	↑	↓	↓	✓	19%	Cost-effective, continuous, particle size control, rapid	High thermal stress	[29]
Spray Freeze Drying	↓	↓	comparable	~	65–98%	Reduce processing time, particle size control	High energy input, not so scalable	[30,31]
Vacuum Drying	↑	↑	↓	~	54–70%	Reduced energy consumption	Very long processing time, quite high thermal stress	[32]
Fluidized Bed Drying	↑	↓	↓	✓	70%	Low cycle time, continuous	Low heat stress, medium cost	[33]
Electrostatic Spray Drying	↑	↓	↓	~	96%	Low thermal stress, particle size control, continuous, low cycle time	Electrical stress, high costs, limited scalability	[34]

* Survival values refer to different studies on *L. casei* or *L. paracasei* and are reported to provide a comparative overview while limiting strain-specific variability. However, operating conditions and cryoprotective formulations should be considered when comparing the results. For freeze-drying, the reported survival range was 97–98%.

4.1. Freeze Drying

Freeze drying (FD), also known as lyophilization, is one of the most widely employed industrial technologies for water removal, primarily due to its effective preservation of sensitive materials by avoiding elevated temperatures [19]. This method is particularly suitable for probiotic bacterial cells as it effectively preserves the viability, structural integrity, and native morphology of the microorganisms. The powder thus produced typically retains the biological activity and chemical composition of the original product [28], and typically exhibits superior and rapid rehydration properties, allowing the material to quickly return to its original state upon reconstitution [9,35]. Despite these clear benefits, the method presents significant trade-offs: it is time-consuming, costly, and may still induce cellular damage in vulnerable strains. Moreover, the dense, compact product often requires a post-drying step of milling or sieving to achieve a marketable, free-flowing powder [1,6].

The conventional freeze-drying process is defined by three sequential stages: freezing, primary drying (sublimation), and secondary drying (desorption). This process is executed using standard freeze-drying equipment, which, regardless of production scale, consists of a vacuum-sealable drying chamber equipped with heating shelves, a condenser for

trapping water vapor, and dedicated instrumentation for monitoring and control. The condenser location varies, being either integrated within the same chamber (single-chamber system) or housed in a separate unit (dual-chamber system) [28].

A schematic representation of the process is shown in Figure 2.

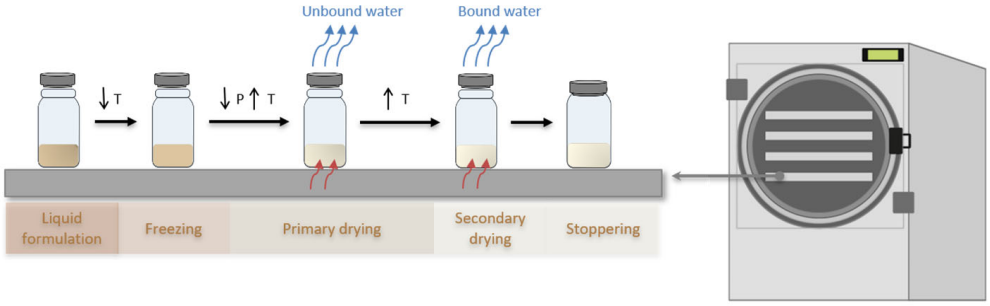

Figure 2. Schematic representation of freeze-drying steps: freezing, primary drying (sublimation), secondary drying (desorption) and stoppering. Original illustration prepared by the authors.

The freezing stage is considered critical as it strongly influences the structure and quality of the final dried product. This initial step, typically conducted at atmospheric pressure, either inside or outside the freeze-drying chamber, involves the crystallization of the solvent (usually water), which separates from the solutes and immobilizes the sample's components. This immobilization is crucial as it helps prevent foaming during the subsequent vacuum step and reduces thermal inactivation [36]. This process is carried out in suitable containers, ranging from small glass vials to large plates or shelves. Freezing protocol profoundly affects the subsequent drying step by dictating the ice crystal size and the resulting pore structure. Rapid cooling produces small ice crystals, whereas slower cooling yields larger ones. Ice crystal size is a key determinant of sublimation dynamics, drying time, and final product characteristics such as porosity, surface area, and rehydration behavior. Specifically, a high ice crystal volume facilitates vapor flow and shortens drying time, while small crystals increase resistance and slow the process. However, excessively large crystals can damage sensitive cellular structures and cause undesirable pH shifts [1,28,36]. Therefore, optimal freezing conditions must be determined case by case, based on the specific formulation.

In the primary drying phase, the frozen solvent is removed via sublimation, where ice transitions directly to vapor under vacuum conditions. Chamber pressure is rigorously reduced below the triple point of water (6.104 mbar; 0.009 °C) to entirely bypass the liquid phase. Typically, working pressures range between 0.03 and 0.2 mbar, depending on product physicochemical properties [37–40]. The driving force for sublimation is the difference in water vapor pressure between the drying chamber and the moving sublimation front. However, as sublimation is an endothermic process, the sample temperature drops, which in turn reduces the ice vapor pressure and consequently slows the drying rate. To compensate, controlled heat input is applied via heated shelves to maintain the required sublimation rate. As sublimation progresses inward from the surface (top-to-bottom in vials or outside-to-inside in spherical samples), a dry, porous matrix forms through which the water vapor must escape. The thickness and porosity of this dry layer significantly affect the resistance to vapor flow and, thus, the overall drying time. Consequently, the duration of primary drying is dependent on both the sample formulation and thickness. Another key control parameter is the sublimation temperature, influencing both the drying rate and the critical factor of bacterial viability preservation [28,36]. The identification of optimal freezing and primary drying conditions is highly strain-dependent and requires

balancing viability, drying time, and final powder quality. These process parameters and their impact on probiotic survival are discussed in more detail in Section 6.2.

Upon completion of primary drying, the product typically still retains non-freezable water. This remaining water is reduced during the secondary drying phase, which is mainly driven by desorption and its strongly influenced by product temperature, matrix composition, cake structure, and mass-transfer resistance. Unlike primary drying, secondary drying is not governed by ice sublimation; depending on product structure, temperature, and formulation, this step may become time-limiting. In this phase, product temperature is generally a key driver of desorption kinetics, while chamber pressure can still influence mass transfer and the driving force for water removal. To accelerate water removal, shelf temperature is increased, provided that the product temperature remains below critical limits related to collapse, glass transition, and biological stability. Additionally, increasing the sample's specific surface area can also help reduce the required drying time. The critical goal of this phase is to reach a residual moisture content and water activity range compatible with the viability retention. Some studies suggest a moisture content lower than 5%, but the appropriate target depends on the probiotic strain, protective formulation, glass-transition properties, packaging, storage humidity, and storage temperature [36,41].

Throughout the drying process, vial stoppers remain partially open to permit vapor release. Once drying is complete and the target residual moisture is achieved, the stoppers are fully inserted to hermetically seal the product, thereby preserving its final state [28]. Alternatively, in bulk freeze drying, the cake formed over trays or containers placed on the shelves is collected, broken into smaller pieces, and transferred into suitable packaging for storage, further handling, such as milling, and subsequent microbiological quality assessments.

4.2. Vacuum Drying

Vacuum drying is an alternative to freeze drying that employs a similar setup, a vacuum chamber with heated shelves and a condenser, but removes water by evaporation while the product remains liquid or semi-liquid on the shelf. Unlike freeze drying, which relies on ice sublimation, vacuum drying operates at higher temperatures and pressures (typically ~30–60 mbar) and is more energy-efficient, with reported savings of ~40% [1,27]. The most critical controls are drying temperature and time, which govern both the final water activity and probiotic viability. Lower temperatures and shorter residence times generally favor microbial survival [1]. Elevated drying temperatures increase membrane damage and, despite lowering final water activity, can compromise cell viability [42]. Overall, vacuum drying offers mild thermal exposure, reduced oxygen contact, and good retention of viability, making it a promising option for sensitive probiotic strains; its principal drawback is the long processing time (≈20–100 h), which depends on formulation and operating conditions [27].

4.3. Spray Drying

Another widely used technique for producing dried probiotic formulations is spray drying (SD), which relies on high-temperature atomization of a liquid cell suspension. This method typically yields spherical, dry powder particles with uniform morphology, acceptable flow properties, and a narrow particle size distribution [27].

A standard spray dryer comprises several essential components: a feed pump, an atomizer, a drying gas source (typically heated air or nitrogen), a drying chamber, and product separation and collection units [43]. The process itself is characterized by four main operational phases: atomization, drying, particle separation, and collection, as outlined in the schematic representation shown in Figure 3.

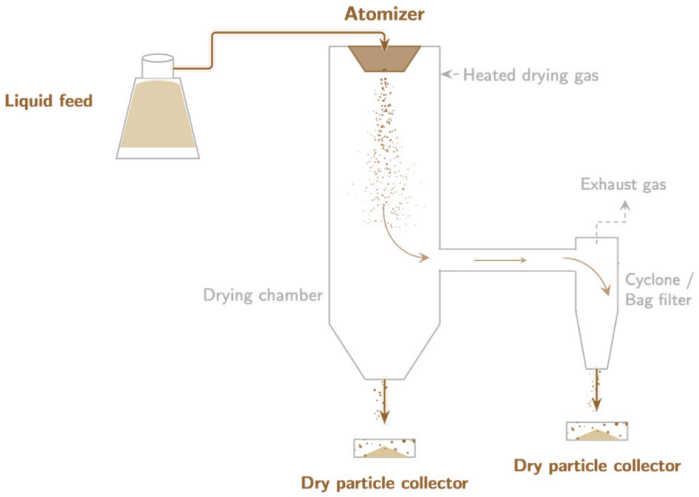

Figure 3. Schematic representation of spray drying process, with its four phases: atomization, drying, particle separation and collection. Original illustration prepared by the authors.

Spray drying begins with the atomization of a liquid feed into a fine mist of droplets. The atomizer is a critical component in this process since its configuration directly influences the resultant particle size and the overall drying efficiency. Four primary atomization technologies are commonly employed, classified according to the energy source employed to generate the spray: (i) Centrifugal atomizers, which rely on a rapidly rotating disk or wheel to mechanically disperse the liquid; (ii) Hydraulic atomizers, where the feed is forced through a narrow nozzle under high pressure; (iii) Two-fluid (kinetic) atomizers, in which compressed air or gas breaks the liquid stream into droplets; (iv) Sonic atomizers, where ultrasonic energy generated within the nozzle disintegrates the liquid into a fine spray [43]. The choice among these atomization technologies is conditional upon the unique advantages of each method and is primarily governed by the desired final powder characteristics and the specific requirements of the formulation.

Once atomized, the spray is introduced into the drying chamber, where it comes into contact with a stream of heated gas, typically air or nitrogen. The temperature of the drying gas is usually between 150–200 °C, and so product temperature increases progressively during drying and depends on coupled heat and mass transfer phenomena, including evaporative cooling, droplet size, feed solids content, gas humidity, residence time, and flow configuration. Depending on the relative orientation of the feed and drying gas, three flow configurations can be used: co-current, counter-current, and mixed flow. Among these, co-current flow is the most widely adopted in probiotic drying. This configuration minimizes heat exposure by ensuring that the wettest droplets encounter the highest temperatures and the driest particles the lowest. This mechanism ensures effective drying while significantly reducing thermal damage to heat-sensitive cells.

The third phase involves the rapid drying of the atomized droplets, quickly leading to the formation of dry powder particles. In the final phase, the separation of solids from the drying gas is achieved. Larger and heavier particles typically settle by gravity and are collected at the bottom of the chamber, while finer particles are efficiently recovered using specialized equipment such as cyclones or bag filters [1].

The physicochemical properties of the final powder are determined by both equipment design (e.g., atomizer type, chamber geometry) and process parameters, which must be carefully tailored for each probiotic strain. Key operating parameters in spray drying include inlet and outlet air temperatures, nozzle type and size, air flow rate and pressure, and feed rate [44,45]. However, these variables should not be interpreted independently, because probiotic inactivation during spray drying results from coupled heat and mass

transfer phenomena occurring at the droplet and particle scale. Inlet gas temperature, outlet temperature, air flow rate, gas humidity, feed rate, feed solids concentration, feed viscosity, atomizer design, droplet-size distribution, flow configuration, and residence-time distribution jointly determine the drying rate, evaporative cooling, particle temperature, final residual moisture content, and the thermal history experienced by the cells. Therefore, the outlet temperature is often a useful process indicator, but it does not alone describe the thermal history experienced by the cells. Higher gas temperatures may increase the driving force for evaporation and shorten drying time, but they can also increase thermal and dehydration stresses once the particle surface becomes dry and evaporative cooling decreases [46–49]. Similarly, atomization pressure and nozzle configuration influence viability not only through mechanical shear, but also by changing droplet size distribution, surface-area-to-volume ratio, drying kinetics, and exposure time [47,50].

Spray drying represents a rapid, energy-efficient, and cost-effective alternative to freeze drying. It can generate fine powders (typically 10–150 μm in diameter) and continuously process large feed volumes, enabling industrial-scale production. In addition, spray drying offers control over particle morphology and can be scaled up in a relatively straightforward manner in terms of time, energy demand, and equipment design. However, the process exposes cells to multiple stressors, including elevated temperatures, dehydration stress, shear forces, and oxidative damage, all of which can compromise viability if not properly managed [1,27,43]. These stresses are interdependent: rapid water removal can reduce the time spent at high temperature, but excessive dehydration or low residual moisture may impair membrane integrity and rehydration capacity, whereas insufficient drying can compromise powder stability during storage. Therefore, careful selection of drying conditions and protective strategies is essential to maintain probiotic functionality in the final product.

Overall, process optimization can substantially improve probiotic survival, but it is inherently strain-specific and often involves trade-offs between viability, cycle time, and final powder stability. A more detailed discussion of how process parameters can be tailored to the specific microorganism and formulation, and how these settings can be optimized, is provided in Section 6.2.

4.4. Spray Freeze Drying

Spray freeze drying (SFD) is an emerging technology for producing biopharmaceutical and probiotic powders, combining features of both spray drying and freeze drying [27]. It enables the production of highly porous particles with large specific surface area and tunable particle size, with powder properties (flowability, hygroscopicity) that depend on formulation composition, solids content, atomization conditions, freezing configuration, and drying parameters [9].

In spray freeze drying, a liquid feed is atomized directly into a cryogenic medium (typically liquid or cold vapor nitrogen), leading to instantaneous droplet solidification. As in conventional spray drying, the selection of atomization technology is critical, since it determines droplet size and distribution, which in turn influence the final powder morphology and stability. Common cryogens include liquid and gaseous nitrogen, although other fluids such as liquid argon, carbon dioxide, propane, and pentane have also been used. Ideal cryogenic media share key properties: low boiling point, chemical inertness, and suitable density and viscosity to promote rapid heat transfer and efficient droplet breakup. Three main configurations are typically used to achieve droplet freezing in spray freeze drying. (i) Spray freezing into vapor (SFV): In this configuration, the atomized solution is introduced into a chamber filled with cold cryogenic vapor (typically nitrogen). Droplets freeze while falling through the vapor phase, avoiding contact with surfaces in the liquid

state. This limits agglomeration, facilitates downstream powder collection and favors cryogen recovery by lowering nitrogen consumption in the early stages of drying [51]. (ii) Spray freezing into liquid (SFL): Here, the droplets are sprayed directly into a cryogenic liquid. Because heat transfer in liquids is faster than in gases, this configuration achieves the highest freezing rates and therefore minimizes phase separation within the droplet. Moreover, it allows flexibility in cryogen selection: nitrogen can be used at atmospheric pressure, whereas pressurized systems permit the use of lower-cost cryogens such as carbon dioxide [51]. (iii) Spray freezing into vapor over liquid (SFV/L): In this setup, the atomized solution is sprayed into a chamber containing boiling cryogenic liquid, which creates a vapor layer above the surface. Partial or complete freezing occurs within this vapor layer before any droplet contacts the liquid, reducing the risk of contamination or structural damage caused by direct liquid–product interaction [51]. These three possible configurations are schematically represented in Figure 4.

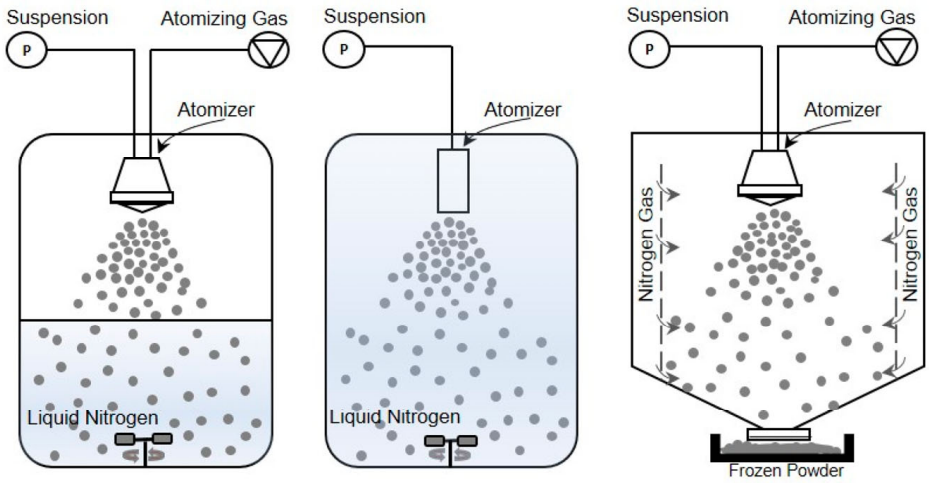

Figure 4. Possible configurations for droplets freezing in spray freeze drying: spray freezing into vapor over liquid (**left**), spray freezing into liquid (**middle**), spray freezing into vapor (**right**). Reprinted with permission from [51].

After freezing, the collected frozen droplets are dried in a freeze dryer to remove solvent and obtain the final powder. The atomization and freezing stages primarily determine the physical and morphological characteristics of the powder, whereas the subsequent drying step determines process time and energy demand. The particle form, morphology, and internal structure are influenced by both formulation properties (e.g., solute concentration and composition) and processing parameters (e.g., temperature, pressure, and ambient humidity) [52]. For example, a study on *L. rhamnosus* indicates that probiotic survival is strongly governed by the combined effect of atomization parameters rather than by any single operating variable. Specifically, coordinated settings (e.g., spray rate and deflection pressure, or spray rate and nozzle orifice size adjusted in a consistent way) were associated with improved viability, whereas mismatched intermediate conditions led to a pronounced loss of cell survival [53].

A major advantage of SFD is its compatibility with continuous processing, in particular with continuous atomization and droplet freezing, which is highly attractive for pharmaceutical manufacturing. However, when frozen particles are subsequently dried in a conventional freeze dryer, the sublimation step remains batch-based unless dedicated continuous freeze-drying equipment is used. Additional benefits include reduced thermal stress on heat-sensitive biomaterials, scalability potential, and the possibility of generating powders with high porosity and large specific surface area. These structural features can favour rapid reconstitution, but they may also lead to low bulk density, powder cohesive-

ness, electrostatic effects, and increased moisture sorption, depending on the formulation and storage conditions. Overall, several limitations remain: high energy consumption, long cycle times, relatively low production yields, powder handling issues, moisture sensitivity and the need to ensure aseptic handling of the resulting powder [9,44,52].

4.5. Electrostatic Spray Drying

Electrostatic spray drying (ESD) is a relatively recent drying technique that represents an alternative for the dehydration of heat-sensitive products. It can support the production of engineered powders with controlled particle size and morphology, and may also contribute to improved properties such as flowability and rehydration behaviour, although these remain dependent on formulation and process conditions.

Electrostatic spray drying involves the application of an electrostatic charge (typically 1–30 kV) to a conductive liquid feed through a charged atomization system, most commonly based on a bi-fluid electrostatic nozzle in the studies reported for probiotic and biopharmaceutical applications [54]. A high-voltage generator supplies the charge, while a pump delivers the liquid formulation to the nozzle. The electrostatic field contributes to droplet formation and produces charged droplets. These droplets are then introduced into a closed drying chamber, where they contact a hot drying gas, similarly to conventional spray drying. This gas is often nitrogen, and it may help limit oxidative stress, as in conventional spray drying. Due to the presence of the electrostatic field, efficient atomization and drying may be achieved at lower inlet gas temperatures than those commonly used in conventional spray drying, depending on the formulation, feed properties, gas flow rate, and equipment configuration [35]. Within the chamber, moisture evaporates into the gas stream, which is continuously removed, and the droplets solidify into charged particles that are subsequently collected in a grounded vessel.

The electrostatic field may also influence droplet internal organization. In some systems, differences in polarity, charge distribution, solubility, and diffusion rates may contribute to a non-uniform distribution of components within the drying droplet, potentially favouring water migration toward the surface and modifying shell formation during drying. The resulting dry particles are then neutralized during collection [55].

Several studies have evaluated the applicability of this technology to probiotics and other biologics. Beaupeux et al. compared *L. rhamnosus* powders produced by freeze drying, conventional spray drying, and ESD, and observed that ESD achieved viability levels comparable to FD at both laboratory and semi-industrial scales while offering the advantage of continuous operation for the atomization and drying-gas contact steps [54]. Jayaprakash et al. further reported that ESD improved storage stability of *L. rhamnosus* compared with FD and SD and found that the applied voltage had limited impact on viability within the tested range [35]. Drying gas temperature emerged as a more critical factor: because the applied charge allows efficient atomization and drying at reduced inlet temperatures, ESD can operate under milder inlet-temperature conditions than conventional spray drying in matched or comparable experimental setups. For probiotic applications, inlet temperatures in the range of 70–90 °C have been shown to limit thermal damage while maintaining high viability in the formulations and operating conditions investigated [35,54–56]. Similar behavior was observed for monoclonal antibody formulations, where ESD at 70 °C produced powders with acceptable residual moisture, a result not achievable with the same spray setup in the absence of an applied charge [57]. However, direct comparison of ESD with conventional spray drying should consider not only inlet temperature, but also outlet or product temperature, residence time, feed composition, process yield, throughput, energy demand, scale-up conditions, and storage stability.

Overall, ESD offers several advantages: continuous processing potential, reduced thermal stress on thermolabile compounds, the possibility of operating under reduced-oxygen atmospheres when nitrogen is used as drying gas, and preservation of volatile and aroma-active compounds through low-temperature drying. Depending on formulation and process conditions, it can also increase yield and support the production of powders with desirable functional properties (e.g., flowability, rapid rehydration). At the same time, ESD presents challenges, including process and equipment complexity, higher capital cost, and unresolved questions around encapsulation efficiency and long-term stability. Moreover, because its industrial track record is comparatively limited relative to FD and SD, an expanded exploratory phase, spanning different strains, biomasses, and formulations, is especially fundamental during development [35,56].

5. Stress During Drying

Probiotic bacteria are subject to multiple sources of stress during the drying phase. Microorganisms can react to stress through two main strategies: (i) synthesis of stress-response and repair proteins that help maintain essential functions, and (ii) transient increases in tolerance or resistance to harmful conditions (e.g., accumulation of protective solutes), which mitigate damage during exposure [25]. The main stress factors associated with drying are:

- Dehydration stress

  Water removal during drying suppresses metabolic activity and slows degradation reactions, but it also threatens structural integrity. Water contributes to stabilization of membranes, proteins, and enzymes; its loss can lead to membrane destabilization, leakage of intracellular components, and loss of enzymatic function. As a result, severe dehydration can compromise membrane integrity and ultimately reduce viability [1,27].

- Osmotic stress

  As water leaves the cell, intracellular solute concentration increases, cytoplasmic volume decreases, and plasmolysis may occur, impairing key cellular processes and ultimately leading to inactivation. Processes such as spray drying and freeze drying increase external osmolality, which further intensifies this effect. Probiotic cells can counterbalance osmotic stress by producing stress proteins and accumulating compatible solutes (e.g., certain sugars and amino acids), which help maintain turgor and protect macromolecular structures. However, this adaptive capacity is strain-dependent, and sustained osmotic stress can still result in marked viability loss [25,27,58].

- Thermal and freezing stress

  Elevated temperatures can denature proteins, disrupt membranes, and damage nucleic acids; in response, cells may induce heat shock proteins to stabilize protein structure and function. Conversely, low temperatures, particularly during freezing, reduce membrane fluidity, perturb nucleic acid processing, and promote ice crystal formation and local increases in osmolarity, all of which can damage the cell envelope. Cold-shock responses can partially mitigate this damage, but tolerance to freezing is highly strain-specific and depends on the duration and severity of exposure. In freeze drying, the freezing step is particularly critical: depending on the strain, formulation, freezing rate, and drying protocol, a substantial fraction of cell damage may occur during freezing, and survival after freezing can influence the outcome of the subsequent desiccation step. In specific formulations and experimental conditions, some studies reported that approximately

60–70% of the cells remaining intact after freezing also remained viable after drying [25,33,58].

- Mechanical/shear stress

  During spray drying, the cell suspension is forced through a nozzle at high pressure, exposing cells to intense shear. Increased nozzle pressure has been associated with significant reductions in viability in several species. For example, *Enterococcus faecium* showed a loss of approximately 4 log cycles for an increase of 1.5 bar, and similar trends have been reported for *L. bulgaricus* and *L. lactis*. By contrast, lower atomization pressures improved survival in certain vaginal strains of *L. acidophilus*. However, this effect is not universal, and some studies observed no significant losses at higher pressures [59]. Overall, shear stress may not always be the primary lethal factor, but it can exacerbate heat-induced damage during drying [1].

- Oxidative stress

  Oxidative stress is particularly relevant for oxygen-sensitive strains. During spray drying, reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide can form due to dissolved oxygen in the feed and in the drying gas. When ROS are generated faster than the cellular antioxidant systems can neutralize them, they can attack membrane lipids, proteins, and nucleic acids, ultimately compromising membrane integrity and causing loss of viability. Some species, including several lactobacilli, exhibit partial oxygen tolerance and may produce antioxidant enzymes, such as superoxide dismutase. Another antioxidant enzyme that can be produced is catalase, but its activity is species- and strain-dependent and is often associated with the availability of exogenous heme or other cofactors. In contrast, many strictly anaerobic probiotics, including most Bifidobacterium, have limited oxidative defenses and are therefore highly vulnerable. Extensive oxidative damage can severely limit survival during drying and subsequent storage, underscoring the importance of minimizing oxygen exposure throughout processing [1,9,25,27].

Each drying technology (freeze drying, spray drying, spray freeze drying, etc.) exposes cells to these stress factors to a different extent, which largely explains the differences in viability and long-term stability observed across processes. However, cell damage is not limited to the drying step itself. Rehydration shock and post-drying exposure to humidity, oxygen, and temperature can further affect viability and are therefore discussed separately in Section 7.

6. Protective Strategies

A detailed understanding of how drying parameters and storage conditions influence bacterial survival and vitality is essential for limiting cell death. During drying, structural and functional damage to the cell wall, cytoplasmic membrane, and proteins can reduce bacterial survivability. Preventing this damage is critical, since loss of membrane integrity and protein function leads to inactivation and ultimately affects both the productivity of the process and the quality of the final dried culture [44]. To enhance cell survival during processing and subsequent storage of probiotic products, various strategies have been investigated. First of all, the optimization of growth and process parameters is essential; then complementary approaches may include exposing cells to sublethal stress during fermentation, incorporating protective excipients, and encapsulating probiotics within protective matrices [25].

6.1. Stress Adaptation

Intrinsic stress tolerance is a key determinant of probiotic robustness during production and preservation. This tolerance can be enhanced through different approaches, including genetic modification, stress adaptation or sublethal conditioning of natural strains, and the selection of inherently resistant strains. Since genetic modification results in genetically modified organisms, the latter two strategies are generally preferred in industrial and regulatory practice [25]. Focusing on stress adaptation, exposing bacteria to controlled challenging conditions during cultivation can activate cellular stress-response pathways and enhance resistance to subsequent processing, thereby improving survival [27,60]. The relevance of stress adaptation is linked to the induction of cross-protective physiological states, in which exposure to mild stress can improve tolerance to subsequent stresses that are not necessarily identical to the initial stimulus. In lactic acid bacteria, cross-protection has been reported after heat, acid, osmotic, oxidative, cold, starvation, or bile-related conditioning, and may improve tolerance to stresses encountered during freezing, drying, storage, rehydration, or gastrointestinal exposure [61,62].

The mechanisms underlying adaptation-induced protection are multifactorial and strongly strain-dependent. Heat adaptation can induce molecular chaperones and proteases involved in protein refolding and removal of damaged proteins, thereby limiting protein denaturation during subsequent thermal or dehydration stress. The role of chaperone systems has been highlighted, for example, in studies on GroESL-overproducing probiotic strains, where enhanced stress tolerance was associated with improved resistance to heat and spray drying [63]. In addition, stress conditioning can promote changes in membrane composition and cell-envelope structure, helping to preserve membrane integrity during dehydration, freezing, or thermal exposure. Osmotic conditioning may favour the accumulation or uptake of compatible solutes, such as amino acids, sugars, or other osmoprotective compounds, contributing to water retention, protein stabilization, and reduced membrane damage. Acid adaptation can modify intracellular pH homeostasis, membrane fatty acid composition, proton transport, and acid-resistance systems, while mild oxidative or aeration stress may enhance antioxidant defenses in strains able to mount this response. These partially overlapping mechanisms explain why a conditioning treatment may improve tolerance not only to the applied stress, but also to other processing-related stresses [61,62,64].

Among the various conditioning strategies, sublethal heat stress is the most widely applied to increase bacterial survival after drying, both in freeze drying and spray drying. Typically, the fermented biomass is exposed to moderately elevated temperatures (approximately 40–55 °C) for a short period (up to 30 min). Multiple studies have demonstrated the beneficial effects of this pretreatment on probiotic viability both immediately after drying and, in some cases, during storage [65–69]. Desmond et al., for example, reported an approximately 18-fold increase in viability after spray drying when cells were pretreated at 52 °C for 15 min. In the same work, other sublethal stresses, such as exposure to hydrogen peroxide, salt, or bile salts during fermentation, were also tested. Although generally less effective than heat shock, these treatments still contributed to improved post-drying survival [63]. More recent studies have also confirmed that heat shock can improve freeze-drying tolerance in specific strains; for example, heat treatment of L. acidophilus at 45 °C for 30 min increased survival after freeze drying and was associated with improved preservation of cell wall and membrane integrity [64]. Osmotic conditioning, typically achieved by adding salts such as NaCl, has similarly been investigated as a means to increase resistance to freezing and freeze drying, and has shown promising effects on post-process viability [69]. Additional adaptive strategies include exposing cultures to acidic environments (pH 3–4) and to mild cold stress (around 10 °C) for defined periods be-

fore drying, both of which have been reported to improve survivability during downstream processing [68].

However, stress adaptation should not be interpreted as universally beneficial. The magnitude and direction of the response depend on the species, strain, growth phase, medium composition, conditioning intensity, exposure time, and recovery conditions before drying. Strain-to-strain variability may reflect differences in membrane lipid composition, cell-wall architecture, stress-regulon organization, chaperone expression, compatible-solute transport, antioxidant capacity, and repair systems. This variability is supported by studies showing that sublethal stress can improve spray-drying survival and storage stability in some lactic acid bacteria, such as *L. plantarum*, while providing limited or even negative effects in other strains under comparable conditions [70]. Similarly, bifidobacteria are often more challenging to stabilize because many strains are strictly anaerobic or oxygen-sensitive and display heterogeneous responses to acid, bile, oxygen, heat, and freeze-drying stresses. Their robustness therefore depends strongly on the species and strain considered, as well as on the availability of appropriate protective formulations and oxygen-control strategies [71–73].

From an industrial perspective, stress adaptation is attractive because it can be integrated upstream during fermentation or biomass conditioning without necessarily changing the final product composition. Nevertheless, it requires careful optimization to avoid reducing biomass yield, prolonging production time, or inducing sublethal injury before drying. Therefore, adaptation treatments should be evaluated together with formulation, drying protocol, storage stability, rehydration behaviour, and post-drying functionality, rather than only through immediate survival after drying.

6.2. Optimization of Process Parameters

Optimization of process parameters represents not merely an additional strategy, but the primary step toward enhancing probiotic survivability. This stage should be systematically addressed using experimental design approaches, such as Quality by Design (QbD) principles and Design of Experiments (DoE) methodologies, which enable the identification of critical process parameters tailored to each strain and drying technology [27].

For freeze drying, both the freezing step and the sublimation (primary drying) step can be tuned. During freezing, the most relevant parameters are freezing temperature and freezing rate. For instance, Zhao et al. investigated the impact of freezing temperature on the survival of *L. brevis* during freeze drying and reported that rapid freezing at −65 °C preserved cell viability better than freezing at −20 °C. This improvement can be attributed to more uniform ice formation and minimized solute concentration [74]. Similarly, Aragon-Rojas et al. compared two cooling rates (0.5 °C/min and 1.5 °C/min) for *L. fermentum* and observed that the faster rate resulted in higher survival, which was attributed to the formation of smaller intracellular ice crystals and a consequent reduction in membrane damage and biochemical disruption [75]. In contrast, Vorländer et al. reported a decrease in *S. cerevisiae* viability at both extremes of freezing rate: ultra-rapid freezing at −196 °C caused mechanical damage associated with intracellular ice formation, whereas slow freezing at −20 °C induced osmotic stress due to excessive solute concentration. This highlights that both excessively fast and excessively slow freezing can be detrimental [76]. Verlhac et al. further showed that *L. casei* viability was maximized at a slower cooling rate (0.5 °C/min), suggesting that rapid dehydration and membrane vesiculation at higher rates compromised survival [77]. Schoug et al. identified an intermediate cooling rate of 2.8 °C/min as optimal for *L. coryniformis*, balancing the risks of osmotic stress at low rates and intracellular ice formation at high ones [78]. Collectively, these apparently conflicting findings indicate that no universal optimal freezing rate exists; rather, the most

suitable freezing profile depends on the balance between intracellular ice formation, osmotic stress, and dehydration kinetics, all of which are strongly strain-dependent. Moreover, the formulation composition plays a critical role, since cryoprotective excipients can modify water mobility, membrane stabilization, and intracellular protection, thereby shifting the optimal cooling conditions. Consequently, freezing optimization should be considered as a combined process-formulation problem rather than as an isolated process parameter [78].

Another strategy to improve probiotic survival during freeze drying is the use of an annealing step. In this approach, after freezing, the product is briefly warmed to a temperature above the glass transition temperature of the freeze-concentrated matrix (Tg'), while remaining below the melting temperature or relevant eutecticlimits, and held there before primary drying. This controlled hold provides sufficient molecular mobility within the freeze-concentrated phase to promote ice-crystal coarsening, matrix relaxation, and solute redistribution. The resulting larger ice crystals create a more open pore structure and facilitate ice sublimation during primary drying. Besides improving drying efficiency, annealing has been reported to enhance post-drying viability retention in strains such as *L. reuteri* and *L. acidophilus*. However, the effect of annealing depends on the formulation, strain, freezing history, annealing temperature, and holding time, since excessive thermal exposure or inappropriate annealing conditions may negatively affect cell survival [38,79].

During sublimation, key process variables include shelf and product temperature, chamber pressure, and total drying time, which are interdependent. Careful selection of these parameters can increase survival and shorten cycle time. Aragón et al. compared sublimation temperatures of −30 °C, −20 °C, and −10 °C and showed that higher sublimation temperatures accelerated ice removal, shortened primary drying, and improved viability. The authors attributed this effect to the reduced exposure time: prolonged drying at lower temperatures can promote conformational changes in proteins, and increase structural damage to the cells. In general, similar studies have reported that running primary drying at slightly higher shelf temperatures can reduce sublimation time by around 40% while maintaining viability, provided that the product temperature remains below the collapse/glass transition limit of the matrix [75,77].

For spray drying, survival is strongly influenced by operating conditions such as inlet and outlet air temperatures, droplet size (governed by nozzle configuration and atomization pressure), and feed flow rate [27]. Temperature is generally considered the dominant factor, but the thermal load experienced by the cells depends on the balance between gas temperature, evaporation rate, evaporative cooling, residence time, and the progressive increase in particle temperature as moisture is removed. For this reason, higher inlet or outlet temperatures are often associated with lower survival, but their effect depends on droplet size, drying time, feed rate, final water activity or residual moisture, and the protective formulation used [9,43,47,48,80]. Techniques such as electrostatic spray drying can mitigate thermal stress by enabling efficient atomization and drying at lower gas temperatures, thereby improving survival [55].

Feed flow rate is another critical parameter. As shown by Alves et al., operating at an optimal feed rate improved survival under the specific conditions of their study. In the study, higher feed rates generated larger droplets, which modify drying kinetics by changing droplet size, heat and mass transfer rates, residence time, and residual moisture in the final powder. Larger droplets may benefit from stronger evaporative cooling and reduced particle heating during the early drying stages, but they may also dry less completely and retain higher moisture. Excess moisture can compromise shelf stability by promoting chemical and biological degradation during storage. Therefore, the effect of feed flow rate should not be generalized, as it depends on atomizer design, feed properties, air-flow configuration, chamber loading, and the target residual moisture [81]. Atomiza-

tion pressure further affects viability. Lower nozzle pressures generally improve survival because they reduce shear forces and mechanical stress on the cells. At the same time, atomization pressure also affects droplet size distribution and surface-area-to-volume ratio, thereby influencing drying rate, exposure time, particle temperature, and final powder moisture. Riveros et al. demonstrated this effect in *L. acidophilus*, highlighting atomization pressure as a key lever for maintaining probiotic viability during spray drying [80].

6.3. Addition of Protective Substances

The addition of excipients such as saccharides, proteins, polyols, and gums is one of the most widely used strategies to protect probiotics during drying processes, particularly in spray drying and freeze drying. These compounds are often described as protectants, carriers, or protective agents, and they act by stabilizing cell membranes and intracellular proteins against dehydration- and ice crystal–induced damage [6,82]. Although the precise mechanisms of protection are not yet fully understood, several complementary hypotheses have been proposed. First, the vitrification theory suggests that excipients form a glassy, highly viscous matrix that immobilizes cellular structures and slows deleterious reactions. Second, the water replacement hypothesis proposes that hydroxyl groups of sugars replace water molecules normally bound to phospholipids and proteins, thereby preserving membrane integrity during drying. Third, the hydration force hypothesis suggests that excipients help maintain appropriate spacing between membrane headgroups under severe dehydration. In addition, excipients may mitigate osmotic stress by promoting the accumulation of compatible solutes or by inducing mild osmotic dehydration prior to drying. They can also contribute to protection by scavenging reactive oxygen species, enhancing thermal resistance via stress-response mimicry, and modulating drying kinetics to achieve gentler water removal [1,82,83].

Low-molecular-weight protectants, such as mono-, di-, and oligosaccharides (e.g., trehalose, sucrose, lactose, maltose, sorbitol), are particularly effective because they readily vitrify and form hydrogen bonds with membranes. High-molecular-weight protectants, including milk proteins, gelatin, inulin, and polysaccharides, tend to form viscous surface layers around the cells, thereby reducing mechanical stress and limiting oxidative damage [9,84]. Certain excipients, notably trehalose and sucrose, act as both cryoprotectants and lyoprotectants, improving probiotic survival during freeze drying and stabilizing cells during subsequent storage [1].

From a functional standpoint, excipients can be grouped into different classes. Stabilizers, mainly sugars such as sucrose and trehalose, preserve protein structure and prevent aggregation during drying. Bulking agents, including mannitol, glycine, and certain amino acids (e.g., phenylalanine, arginine), provide physical support, improve cake appearance, and promote an appropriate pore structure. Surfactants, such as polysorbates, limit interfacial stress and reduce protein and cell adhesion to air–liquid interfaces, while buffers maintain pH stability throughout processing. Recent studies have also explored novel additives such as polyethylene glycol (PEG) and cyclodextrins, which can enable faster drying at relatively higher temperatures, although their compatibility and effectiveness require careful evaluation [41].

Among the several excipients that have been investigated to improve probiotic survival during drying, carbohydrates are the most frequently reported class. Disaccharides such as sucrose, trehalose, and lactose are widely used, particularly in freeze drying, because of their ability to stabilize cellular membranes and proteins through water replacement and glass formation. Among them, sucrose and trehalose are the most commonly studied and are often associated with improved survival after drying, although their effectiveness depends strongly on the bacterial strain, concentration, and drying condi-

tions. Polysaccharides and prebiotic compounds, including inulin, maltodextrin, starch derivatives, fructo-oligosaccharides, and galacto-oligosaccharides, have also been used either alone or in combination with sugars, acting mainly as matrix-forming agents and carriers able to modulate water removal, glass transition properties, and storage stability. Protein-containing matrices, such as skim milk, whey proteins, and other dairy-derived components, can provide additional protection through film formation, buffering capacity, and interactions with the bacterial surface. Overall, the available studies indicate that no single excipient is universally optimal across probiotic strains and drying technologies. Rather, protective performance is highly formulation- and process-dependent, and the strongest effects are often observed when excipients are combined to exploit complementary mechanisms, such as vitrification, membrane stabilization, matrix formation, and antioxidant protection. A detailed summary of the excipients most commonly reported in the literature, including probiotic species or strain, drying technique, concentration and concentration basis, reported protective effect, and reference, is provided in Supplementary Table S1. Because the studies differ in formulation basis, drying protocol, viability endpoint, and storage conditions, these data should be interpreted as a qualitative or semi-quantitative evidence map rather than as a direct cross-study ranking [35,38,74,77,85–107]. Among the several excipients that have been investigated to improve probiotic survival during drying, carbohydrates are the most frequently reported class. Disaccharides such as sucrose, trehalose, and lactose are widely used, particularly in freeze drying, because of their ability to stabilize cellular membranes and proteins through water replacement and glass formation. Among them, sucrose and trehalose are the most commonly studied and are often associated with improved survival after drying, although their effectiveness depends strongly on the bacterial strain, concentration, and drying conditions. Polysaccharides and prebiotic compounds, including inulin, maltodextrin, starch derivatives, fructo-oligosaccharides, and galacto-oligosaccharides, have also been used either alone or in combination with sugars, acting mainly as matrix-forming agents and carriers able to modulate water removal, glass transition properties, and storage stability. Protein-containing matrices, such as skim milk, whey proteins, and other dairy-derived components, can provide additional protection through film formation, buffering capacity, and interactions with the bacterial surface. Overall, the available studies indicate that no single excipient is universally optimal across probiotic strains and drying technologies. Rather, protective performance is highly formulation- and process-dependent, and the strongest effects are often observed when excipients are combined to exploit complementary mechanisms, such as vitrification, membrane stabilization, matrix formation, and antioxidant protection. A detailed summary of the excipients most commonly reported in the literature, including probiotic species or strain, drying technique, concentration and concentration basis, reported protective effect, and reference, is provided in Supplementary Table S1. Because the studies differ in formulation basis, drying protocol, viability endpoint, and storage conditions, these data should be interpreted as a qualitative or semi-quantitative evidence map rather than as a direct cross-study ranking [35,38,74,77,85–107].

In many cases, combinations of excipients yield synergistic effects, performing better than individual components [44]. For example, pairing sugars with proteins can generate composite protective matrices that both lower water activity and physically shield the cells, resulting in higher survival after drying and improved long-term stability. Depending on the specific probiotic strain and the dairy or food matrix, successful formulations often combine oxidation inhibitors (e.g., ascorbates), water replacement agents (e.g., trehalose, sucrose), and membrane stabilizers (e.g., dairy proteins, phospholipids) [108]. The addition of micellar casein to sucrose, for instance, has been reported to significantly enhance probiotic survival [109]. Such multicomponent systems are frequently optimized using

statistical design tools such as response surface methodology, although careful balancing of component ratios is required, since excessively high concentrations or unfavourable interactions can become destabilizing rather than protective [107]. Additional examples of composite formulations optimized to enhance viability are reported in Table 2. Overall, the protective effect of a given formulation is highly strain-dependent, and optimal compositions generally need to be determined empirically.

Table 2. Composite (multicomponent) formulations optimized to enhance probiotic viability during drying, listed by strain, design of experiments (DoE), optimized formulation components and ratios.

Species	DoE Design	Optimal Formulation	Reference
L. plantarum	Screening design	Sucrose (6.67% *w*/*v*) + inulin (6.67% *w*/*v*) + skim milk (6.67% *w*/*v*)	[92]
L. rhamnosus	Fractional factorial design	Sucrose (10% *w*/*v*) + isomaltooligosaccharide (2.5% *w*/*v*) + hydroxyproline (12% *w*/*v*)	[86]
L. rhamnosus	Box–Behnken	Trehalose (11.07%) + glycerine (9.14%) + sodium glutamate (3.45%) + skim milk (15.74%)	[106]
L. salivarius	Central composite design	Skim milk (9.85% *w*/*v*) + sucrose (10.65% *w*/*v*)	[100]
L. lactis	Screening design	Galactose (10% *w*/*v*) + trehalose (10% *w*/*v*)	[101]
L. lactis	Response surface methodology	Trehalose (4.2%) + mannitol (2%) + skim milk (11.9%) + glutamic acid monosodium (4.1%)	[107]
S. cerevisiae	Central composite design	Skim milk (6.5–10%) + maltose (1.8–4.5%) + maltitol (16.5–18.2%)	[104]
Multiple strains	Screening design	Skim milk (10%) + trehalose (5%) + ascorbic acid (2%)	[110]
Multiple strains	Screening design	Trehalose (8% *w*/*v*) + sodium ascorbate (4% *w*/*v*) + skim milk (6% *w*/*v*)	[85]

6.4. Encapsulation

Encapsulation is widely used to improve the survival, stability, and delivery of probiotics in food and pharmaceutical systems. This approach aims to protect bacterial cells against environmental stresses such as low pH, bile salts, oxygen, freezing, or heat exposure. However, the term "encapsulation" is often used in the literature to describe different strategies that should be distinguished. Microencapsulation refers to the entrapment of probiotic cells within discrete particles, typically ranging from a few micrometres up to approximately 1 mm, in which the cells are retained within a liquid or solid core, membrane, or polymeric matrix. Matrix embedding describes the dispersion of cells within a continuous protective solid matrix, as commonly observed when carbohydrates, proteins, or other carrier materials are used during drying. Coating refers to the deposition of one or more external layers around cells or cell-loaded particles, whereas immobilization implies the retention of cells within or onto a carrier structure, often with limited cell release. These approaches should also be distinguished from carrier-assisted drying, which refers to the use of protective excipients or carrier materials to support drying and improve cell survival without necessarily forming a well-defined capsule, as discussed in the previous section [6,111,112].

Encapsulating materials can be grouped into natural and synthetic matrices. Natural matrices, which usually are characterized by their biocompatibility, biodegradability and safety, are based on polysaccharides or proteins. Polysaccharide-based systems, including alginate, κ-carrageenan, starch derivatives, gum arabic, gellan, xanthan, pectin, and chitosan, are among the most commonly used matrices because of their biocompatibility, film-forming ability, gelation properties, and suitability for food applications. Protein-based matrices, such as gelatine, milk proteins, whey protein isolate, casein, and plant-derived proteins, can also provide protection through film formation, water re-

placement, and interactions with the bacterial surface. Composite systems combining polysaccharides and proteins are particularly attractive because they can improve mechanical resistance, barrier properties, and release behaviour compared with single-component matrices [25,44,84,112–114]. Synthetic biodegradable polymers such as PLA, PGA, PEG, and PVA have also been explored for tailored release profiles and improved stability. However, their suitability should be qualified according to the intended application, since these materials are more commonly associated with pharmaceutical, biomedical, or controlled-delivery systems, whereas food-grade probiotic products generally require matrices with established safety, regulatory acceptance, and sensory compatibility [113]. Importantly, encapsulation efficiency and protective performance are highly strain-dependent. For instance, when high-amylose maize starch, maltodextrin and gum arabic were compared as spray-drying carriers, high-amylose maize provided the greatest protection for *L. plantarum*, whereas maltodextrin and gum arabic were more effective for *L. acidophilus* [14,115].

The protective effect of encapsulation during drying is related not only to the physical barrier formed around the cells, but also to the interactions established between the cells and the surrounding matrix. Encapsulating materials can reduce dehydration stress by creating a more gradual local change in the cell microenvironment. Carbohydrate- and protein-based matrices may also contribute to membrane and protein stabilization through water replacement, hydrogen bonding, and vitrification, thereby limiting membrane phase transitions, protein denaturation, and oxidative damage during drying and subsequent storage. However, the relative contribution of the different protective effects depends on the strain, matrix composition, drying technology, residual moisture, and storage conditions.

Multiple encapsulation techniques are available, each with specific advantages and constraints. Drying-based methods are particularly relevant for probiotic manufacturing because they combine stabilization and powder production. Spray drying is the most widely used and scalable approach, producing inexpensive powders but exposing cells to high thermal stress; electrostatic spray drying is a newer variant that applies an electric field to reduce droplet size and drying time, as shown for *L. plantarum* encapsulated with starch, maltodextrin and trehalose, which achieved slightly higher survival than conventional spray drying [46]. Freeze drying avoids thermal damage and generates porous matrices that enhance storage stability, albeit with longer cycles and higher cost. Spray-freeze drying combines atomization with rapid freezing and sublimation to yield fine, low-density powders with excellent protection; for example, *L. plantarum* encapsulated in whey protein isolate plus sodium ascorbate showed particularly good encapsulation efficiency and storage stability with SFD [116]. Other studies with *L. plantarum* encapsulated in whey protein isolate, polyglycerol polyricinoleate and pectin reported good efficiencies and survival with both spray drying and freeze drying [117].

Beyond drying-based approaches, extrusion, emulsification/internal gelation, ionic gelation and complex coacervation have also been explored as encapsulation technologies. Extrusion involves dropping a polymer–cell suspension into a crosslinking bath to form beads, whereas emulsification/internal gelation produces microbeads within an oil–water emulsion. Ionic gelation relies on rapid crosslinking between charged polymers and multivalent ions, while complex coacervation is based on phase separation between oppositely charged biopolymers. For instance, *L. salivarius* encapsulated in high-polymerisation-degree agave fructans combined with sodium alginate via ionic gelation achieved encapsulation efficiencies above 90% [118]. Other options include liposomes or bilayer entrapment and layer-by-layer polyelectrolyte coatings, which can be combined with a secondary drying step to obtain stable powders. Emerging technologies such as fluidized-bed coating, supercritical fluid encapsulation, and micro/nanoparticle spray congealing are also being explored to improve gastrointestinal targeting and shelf-life [119].

Collectively, these techniques underscore that careful matching of encapsulating matrix and processing method can strongly influence probiotic survival during drying, storage, and subsequent gastrointestinal transit.

When comparing encapsulation strategies, it is important to clearly distinguish the main performance indicators. Encapsulation efficiency generally refers to the fraction of cells successfully entrapped or associated with the encapsulating matrix relative to the initial cell amount. This parameter should not be confused with cell recovery after processing, which describes the number of viable cells recovered after encapsulation and/or drying, or with drying yield, which refers to the mass of dried product recovered relative to the initial feed solids. Similarly, survival rate describes the fraction of viable cells retained after a specific stress, such as drying, storage, or simulated gastrointestinal exposure. Other relevant parameters include cell loading, usually expressed as viable cells per unit mass of carrier or dried product, particle size distribution, release conditions, and storage performance. Reporting these parameters separately is essential, because a system may show high encapsulation efficiency but poor survival after drying, or good immediate recovery but limited stability during storage.

A critical parameter influencing the performance, durability, and efficacy of probiotic encapsulation systems during processing, storage, and gastrointestinal transit is particle size. Larger particles may provide a thicker diffusion barrier against oxygen, gastric acid, and bile salts, thereby improving cell protection under harsh conditions. However, they may also negatively affect mouthfeel, dispersibility, and incorporation into food products. Conversely, smaller particles are generally more compatible with food applications and may improve product homogeneity, but their reduced barrier thickness can limit protection during gastric and intestinal exposure. Because microbial cells are relatively large, they cannot be effectively encapsulated within excessively small particles. Consequently, several studies have identified an optimal particle size range of approximately 100–200 μm, representing a compromise between protection and product performance [113,120]. Therefore, particle size should be interpreted together with other key characteristics, including matrix composition, porosity, cell loading, mechanical resistance, and release behaviour. For example, higher surface porosity is generally associated with faster release of bioactive compounds from microcapsules [120].

Encapsulation not only increases cell viability during drying and storage but can also enable controlled or targeted release in the gastrointestinal tract, where survival through gastric acidity and bile exposure is critical [6]. The addition of cryo- or osmo-protectants to the encapsulating matrix, as well as secondary coatings (e.g., chitosan over alginate beads), can further enhance protection. These systems may also confer a potential for colon targeting; however, such functionality cannot be assumed solely based on the biodegradability or mucoadhesive properties of the polymers used and should be supported by appropriate evidence, such as release studies under simulated gastrointestinal conditions, dynamic digestion models, or in vivo data. For instance, *L. acidophilus* protected with sucrose showed markedly higher survival when entrapped within alginate–chitosan beads [25,88,121].

Although encapsulation has repeatedly been shown to significantly improve the survival of lactobacilli and bifidobacteria in fermented milks and functional food products, thereby supporting probiotic functionality across processing, storage, and gastrointestinal transit, this technique also presents important limitations [122]. Direct quantitative comparison between encapsulation systems remains difficult because studies often differ in strain, initial cell concentration, matrix-to-cell ratio, particle size, drying conditions, storage temperature, relative humidity, and viability endpoint. The inclusion of an extra formulation or processing step can increase production costs, process complexity, and the risk of cell losses before or during drying. In addition, encapsulated systems may require

more careful control of storage conditions, since the protective effect depends on matrix stability, residual moisture, and interactions between the cells and the carrier material. Another critical aspect is release behaviour: while stronger encapsulation can improve protection during processing, storage, and gastric passage, it may also delay or limit cell release at the intended site of action. Therefore, encapsulation systems should be evaluated not only in terms of immediate survival, but also considering process yield, storage stability, release performance, sensory impact, regulatory suitability, and compatibility with the final product [113,123].

7. Post-Drying Stability, Shelf Life, and Rehydration Performance

Post-drying storage stability is a critical determinant of the industrial relevance of dried probiotic products [21]. High survival immediately after drying does not necessarily translate into long-term stability, and viable cell counts alone do not fully describe probiotic quality. During storage, cells may progressively lose culturability, membrane integrity, metabolic activity, or strain-specific functional properties, depending on the formulation, drying history, and environmental conditions. Therefore, post-drying performance should be evaluated not only in terms of immediate survival, but also considering viability loss over time, physiological state, and, where relevant, functional properties such as acid and bile tolerance, adhesion ability, antimicrobial activity, metabolic activity, or other strain-specific effects [124].

The main factors affecting storage stability include storage temperature, water activity, residual moisture, oxygen exposure, and packaging properties. Storage temperature is one of the most relevant parameters, since higher temperatures generally accelerate viability loss and degradation kinetics. In amorphous protective matrices, stability is strongly influenced by the relationship between storage temperature and glass transition temperature (Tg): maintaining the storage temperature sufficiently below Tg is generally associated with reduced molecular mobility and improved retention of probiotic viability. However, the glassy state alone does not guarantee long-term preservation, which depends on the combined optimization of formulation, residual moisture, water activity, packaging, and storage conditions [125–127]. Water activity and residual moisture also play a central role. Moisture uptake can plasticize the solid matrix, lower Tg, and increase molecular mobility, thereby reducing the ability of the matrix to immobilize cellular structures during storage. Residual moisture must therefore be carefully controlled, since insufficient drying may promote molecular mobility and degradation reactions, whereas excessive dehydration can compromise biological activity by removing the minimal hydration layer required to preserve membrane and protein structures [127]. Moisture-induced plasticization may also promote time-dependent physical changes in amorphous powders, including structural relaxation, stickiness, caking, and crystallization of amorphous components. In particular, crystallization can release sorbed water, locally increase water activity, and further accelerate deteriorative reactions [127,128]. Finally, packaging properties are critical to limit both moisture uptake and oxygen exposure. Powders that are initially stable after drying may undergo progressive viability loss if exposed to humid environments or stored in packaging with insufficient water-vapor barrier properties. In addition, oxygen exposure can promote oxidative damage, particularly in oxygen-sensitive strains, making packaging permeability, headspace composition, vacuum or nitrogen flushing, oxygen scavengers, and desiccants important tools in shelf-life design [21,128–133].

Drying technology can also affect storage stability by determining the physical structure of the final powder. For instance, Poddar et al. compared the storage stability of *L. paracasei* powders produced using different drying technologies and evaluated their behaviour at different water activity levels. In that specific study, fluidized bed drying

provided the best protection during storage, which was associated with lower product porosity and smaller agglomerate size. These structural features may reduce water uptake and help maintain a more rigid matrix, thereby limiting moisture-induced mobility and detrimental degradation phenomena [134]. Cross-technology comparisons of storage performance should therefore be interpreted cautiously, because viability loss, residual moisture or water activity, powder structure, packaging, analytical method, and storage conditions are often not matched across studies. Overall, storage stability should be interpreted as the outcome of multiple interacting factors rather than as a direct consequence of a single parameter, such as Tg, water activity, packaging atmosphere, or drying technology alone [125,127].

From a methodological perspective, shelf-life evaluation should distinguish between real-time and accelerated storage studies. Storage stability should ideally be described in terms of degradation kinetics, such as log viability loss over time under defined storage conditions, rather than only as survival at a single endpoint. Accelerated tests at elevated temperature or relative humidity are useful for rapid formulation screening and for comparing degradation trends, but they may not always reproduce the mechanisms occurring under intended storage conditions [133]. Real-time studies remain necessary to confirm shelf-life performance, define appropriate storage conditions, and estimate the overage required to guarantee the target viable count until the end of shelf life. Such overage should be defined from validated stability data, since immediate post-drying survival alone does not predict the rate of viability loss during storage.

Rehydration represents another critical step in the post-drying performance of probiotic powders. Even when cells survive drying and storage, their recovery and functionality after administration may be strongly influenced by the rehydration step, which determines the transition from a dry, dormant state to a hydrated and metabolically active state. During rehydration, cells are exposed to rapid changes in physicochemical conditions, and inadequate reconstitution may lead to poor viability and reduced final survival. Osmotic shock is considered one of the main causes of damage during rehydration, but cell recovery can also be affected by the composition of the rehydration medium, osmolarity, powder-to-liquid ratio, rehydration rate, temperature, time, and the specific microorganism [135]. The choice of rehydration matrix or excipients is therefore important to preserve cell integrity and support the restoration of probiotic functionality. In this context, Arellano-Ayala et al. showed that rehydration of lyophilized *L. plantarum* with a formulation containing sugars, salts, and non-protein nitrogen compounds improved survival under simulated gastrointestinal conditions compared with rehydration in distilled water, and also affected functional properties such as adhesion and cell surface charge [135]. Therefore, post-drying performance should not be assessed only by viable counts immediately after storage, but also by considering the ability of cells to recover physiological activity and retain relevant functional traits after rehydration and exposure to the intended application conditions. This is particularly relevant for probiotic products, since the required viable dose should be maintained until the end of shelf life and delivered in a form able to support the expected health benefit [21].

8. Viability Assessment

As noted above, probiotic products must contain a sufficient number of viable cells to confer health benefits to the host. Viability assessment is therefore crucial not only for quality control and regulatory purposes, but also during processing, where it serves to monitor and optimize conditions that sustain productivity, cell robustness, and stability of the final product [136]. Before comparing analytical methods, it is important to distinguish the different endpoints commonly associated with probiotic viability. In this review, viabil-

ity is used as a broad term referring to the capacity of cells to remain alive and potentially functional under defined conditions. Culturability refers more specifically to the ability of cells to replicate and form colonies under the selected culture conditions, and is therefore the endpoint measured by plate count methods. Vitality describes the physiological activity of cells, including metabolic activity, enzymatic activity, membrane potential, or energy status, without necessarily implying immediate colony formation. Membrane integrity refers to the preservation of the cytoplasmic membrane as assessed, for example, by permeability dyes, whereas metabolic activity refers to measurable biochemical functions such as esterase activity, acidification capacity, respiration, or heat production. Finally, probiotic functionality refers to the ability of a strain to exert its intended health-related effect, which cannot be inferred from viability alone and generally requires strain- and indication-specific functional evidence. Therefore, the different methods presented in this work should not be considered interchangeable estimates of viable probiotic cells, because they quantify different biological attributes. Because each method measures a different endpoint, the choice of the analytical approach should be guided by the specific question being addressed, such as regulatory enumeration, process monitoring, physiological characterization, strain tracking, or functional assessment.

Sample preparation is also a critical pre-analytical step in probiotic viability assessment, particularly for dried, encapsulated, or multi-component products. Rehydration medium, temperature, duration, osmolarity, oxygen exposure, mixing intensity, cell disaggregation, and clump handling can affect both cell recovery and the physiological state measured during analysis. Inadequate rehydration may induce osmotic or membrane damage, whereas excessive mixing or harsh disaggregation may further injure stressed cells. Conversely, insufficient disaggregation can lead to cell clumping, causing underestimation in plate counts, where multiple cells may form a single colony, and inaccurate event detection or gating in flow cytometry. For complex matrices, additional preparation steps may be required, but these should be validated to avoid selectively removing, damaging, or altering the target cells. Therefore, sample preparation conditions should be reported and standardized together with the analytical method.

To support comparison among analytical approaches, Table 3 summarizes the main endpoint measured by each method, together with throughput, repeatability, principal advantages, and major limitations or confounders, including strain and matrix specificity where relevant.

Methods for viability assessment are categorised into two broad categories: (i) culture-dependent methods, which quantify cells relying on their capacity to replicate under defined culture conditions, and (ii) culture-independent approaches, which infer viability from cellular properties other than growth (e.g., membrane integrity, metabolic activity, or nucleic acid signals).

Table 3. Comparative overview of analytical methods used for probiotic viability assessment.

Method	Main Measured Attribute	Throughput	Repeatability	Advantages	Limitations
Plate count enumeration	Culturability	Low	Moderate	Simple, inexpensive, widely accepted, regulatory standard	Time-consuming; affected by culture conditions, dilution errors, and cell clumping; does not detect non-culturable cells
Other culture dependent methods	Growth capacity, replication kinetics, metabolic heat or acidification activity	Low-moderate	Moderate	Useful when plating is difficult; can provide functional information on growth or acidifying capacity	Probabilistic or indirect estimates; affected by medium selectivity, starting cell concentration, turbidity, buffering capacity, matrix opacity, and non-target microbial activity
Fluorescence flow cytometry (FFC)	Membrane integrity, esterase activity, membrane potential, and cell subpopulations	High	High	Rapid, high throughput, single-cell resolution, good repeatability when standardized	Limited species specificity in mixed matrices; affected by dye choice, staining protocol, debris, autofluorescence, clumps, and gating strategy
Impedance flow cytometry (IFC)	Electrical properties related to size, membrane integrity, and intracellular composition	High	High	Label-free, rapid, single-cell analysis	Requires sufficient particle concentration and strain-specific gating; affected by debris, clumps, and medium conductivity
qPCR/dPCR	Target DNA copy number for species or strain quantification	High	High	Highly specific and sensitive; useful in mixed cultures	DNA can persist after cell death; affected by extraction efficiency, inhibitors, gene copy number, and dynamic range
Viability PCR/vPCR	DNA signal mainly from membrane-intact cells after PMA/EMA treatment	Moderate-high	Moderate-high	Combines molecular specificity with intact cell selectivity	Strongly protocol-dependent; affected by dye penetration, light activation, matrix shielding, and membrane state
Single-cell Raman spectroscopy (SCRS)	Label-free biochemical fingerprint, metabolic activity, and identity when coupled with isotope probing or spectral libraries	Low-Moderate	Variable	Non-invasive, label-free, potentially combines vitality and identification	Requires advanced instrumentation, spectral databases, validation, and data analysis; limited routine throughput

8.1. Culture-Dependent Methods

Culture-dependent techniques, which have long been the standard for evaluating the viability of probiotic cultures and products, measure the number of cells that can replicate and form colonies under specific conditions. However, these methods can underestimate the total fraction of physiologically preserved cells by failing to detect cells that retain some biological activity but do not form colonies under the applied culture conditions. Under processing, storage, or host-associated stresses, some bacterial cells may lose culturability while maintaining selected viability-associated features, such as membrane integrity or metabolic activity. This condition is commonly discussed in relation to the viable but non-culturable (VBNC) state, although the assignment of cells to this state requires evidence that loss of colony formation is accompanied by independent indicators of viability. Although culture-based assays are relatively straightforward, they are time-consuming and require careful selection of media and incubation conditions tailored to each specific strain. Since media are inherently selective a formulation suitable for one genus, or even one strain, may be suboptimal for another. Effective medium selection must account for nutrient requirements, oxygen tolerance, and relevant resistances or sensitivities (e.g., to antibiotics) [137,138]. For these reasons, culture-based enumeration methods may be less suitable for complex probiotic blends or for timely monitoring during process optimization.

8.1.1. Plate Count Cell Enumeration

Plate count enumeration remains the conventional and widely accepted method for assessing the viability of probiotic products. In this assay, the sample is serially diluted in a suitable buffer or medium and applied to agar plates, either mixed with molten agar (pour-plate) or spread onto solidified agar (spread-plate). For reliable counts, protocols must be tuned at each step: sample preparation (rehydration/thawing) to preserve cell state, dilutions (homogenization, diluent/media) to ensure representativeness, and plating (media and incubation) to support growth [139]. After incubation under specific conditions, colonies are counted to estimate viable cells in the original sample, with results expressed as colony-forming units (CFU). This approach is technically simple, broadly accepted, and supported by extensive historical data in microbiological viability studies.

Nonetheless, plate count methods have important limitations. They are time-consuming, often requiring more than 72 h for results, and have low throughput. Their precision can be limited, with potential errors up to 35% arising from media variability, dilution inaccuracies, and cell clumping, when multiple cells may form a single colony. Moreover, plate counts detect only colony forming cells and therefore miss viable-but-non-culturable cells, whose prevalence can increase after stress (e.g., freeze drying), leading to underestimation of true viability. Furthermore, the CFU enumeration cannot identify dead cells and is thus unsuitable for evaluating postbiotic products composed of inactivated bacteria. Despite its limitations, plate count cell enumeration remains the industry standard because of its practicality, long-standing use and regulatory acceptance [138,140].

8.1.2. Other Methods

In addition to plate count enumeration, several alternative culture-dependent techniques can assess probiotic viability. The Most Probable Number method estimates viable cells from growth/turbidity patterns in serially diluted liquid cultures using probabilistic models and is useful when plating is hindered by complex matrices. Growth curve analysis tracks optical density over time to infer replication capacity via parameters such as lag phase and maximum growth rate, although outcomes depend on standardizing the starting concentration. Acidification-rate assays, commonly used for lactic acid bacteria, follow pH decline due to lactic acid production and can reflect metabolic activity when plate counts

are unreliable. Isothermal microcalorimetry continuously measures heat flow from metabolizing cells, providing sensitive, real-time readouts with minimal sample preparation, but it lacks species specificity and requires careful calibration. Finally, the Direct Viable Count method distinguishes replicating from non-replicating cells by microscopy after incubation with nutrients and a division inhibitor; it is rapid but requires skilled interpretation. These approaches can complement or, in some cases, substitute plate counting, particularly for stress-exposed probiotics or complex matrices [138].

8.2. Culture-Independent Methods

Non-culture-based techniques have been developed over recent decades to address the limitations of culture-dependent assays. These methods adopt a broader, functional definition of viability, encompassing metabolically active cells and/or cells with intact membranes, and assess viability directly or indirectly by probing membrane integrity, metabolic activity, or nucleic acid content [137,140]. In the following, three complementary culture-independent approaches are described: flow cytometric methods (single-cell resolution of live/injured/dead subsets), molecular methods (PCR/dPCR-based, including viability assays), and single-cell Raman spectroscopy (label-free biochemical fingerprints of viability and identity).

8.2.1. Flow Cytometric Methods

Flow cytometry (FC) is a powerful single-cell analysis technique used to probe the physiological and biophysical properties of microbial cells.

Fluorescence flow cytometry (FFC) enables rapid, high-throughput quantification and discrimination of live, injured, and dead cells based on light scattering and fluorescence signals [141]. This assay typically employ fluorescent dyes targeting specific cellular functions such as membrane integrity, metabolic activity, and membrane potential. Common viability stains include Propidium Iodide (PI), SYTO 9, 5,6-carboxyfluorescein diacetate (cFDA), TOTO-1, 3,3′-Diethyloxacarbocyanine Iodide, and 4′,6-diamidino-2-fenilindolo (DAPI). For instance, PI penetrates only cells with compromised membranes and emits red fluorescence, whereas SYTO 9 labels all cells and fluoresces green. Their combined use enables effective live/dead discrimination. Another example is fluorogenic substrates like cFDA that are hydrolyzed by intracellular esterases in viable cells, producing green fluorescence as an indicator of metabolic activity [142,143].

During measurement, the cell suspension is focused on a sheath fluid to ensure single-cell interrogation as it traverses the excitation beam, typically a 488 nm blue laser. Forward and side scatter provide information on size and granularity, while fluorescence channels report physiological status [143]. Digital event data are then gated to quantify subpopulations; results are frequently expressed as active fluorescent units (AFU), non-active fluorescent units (n-AFU), and total fluorescent units (TFU), corresponding to viable, damaged, and total cells, respectively [136].

Flow cytometry has gained recognition in microbiology, particularly with the implementation of ISO 19344 (IDF 232:2015) [144], which standardizes FFC for the enumeration of lactic acid bacteria and probiotics in fermented products. The ISO method provides three distinct staining protocols, including dual-dye approaches that combine propidium iodide with either a cell-permeant nucleic acid dye (such as SYTO® 24 or similar alternatives) or an esterase-activity probe, allowing the assessment of membrane integrity and metabolic activity in Lactobacillus and Bifidobacterium [145]. An example of a two-colour flow-cytometry gating strategy based on thiazole orange (TO) and propidium iodide (PI) staining is shown in Figure 5, highlighting the discrimination of live, injured, and dead subpopulations according to membrane integrity.

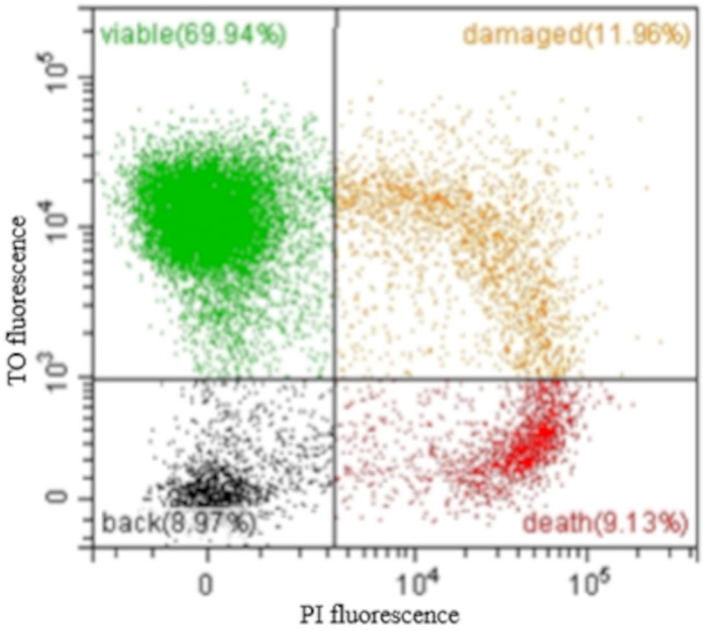

Figure 5. Representative two-colour flow-cytometry dot plot based on thiazole orange (TO) and propidium iodide (PI) staining. Cells exhibiting high TO fluorescence and low PI fluorescence are classified as viable, whereas cells positive for both dyes are considered damaged. Cells with low TO fluorescence and high PI fluorescence are classified as dead. Events negative for both fluorochromes generally correspond to background signals or cellular debris.

Moreover, fluorescence-activated cell sorting (FACS) can further extend FFC capabilities by physically isolating targeted subpopulations, including damaged or non-culturable but physiologically active cells, for downstream analyses and resuscitation studies [137].

Comparative studies generally show good agreement between fluorescence flow cytometry and conventional plate counts, with FFC offering higher repeatability, greater precision, and more robust readouts [145]. Moreover, single-cell analyses indicate that, in freeze-dried populations, loss of cultivability can precede loss of membrane integrity—consistent with the presence of membrane-intact but non-culturable subpopulations that are not captured by plate count method [146].

Nevertheless, FFC has certain limitations. A major concern is its lack of species specificity, particularly in complex food matrices where it may detect non-target microorganisms. Autofluorescence and interference from food particles can also complicate analysis, necessitating careful sample preparation such as filtration or clarification. Moreover, accurate differentiation between VBNC and truly dead cells requires optimized staining protocols and remains technically challenging. Broad adoption of this technique will depend on further standardization, validation across matrices and strains, and regulatory acceptance of FC-based viability metrics [142].

Impedance flow cytometry (IFC) is an emerging, complementary single-cell technique. It measures frequency-dependent electrical impedance as cells traverse microfluidic channels with embedded electrode arrays, yielding multiparametric readouts related to size, membrane integrity, and intracellular composition. A recent comparative study between FFC and IFC demonstrated strong agreement in the quantification of live and active cells across six individual probiotic strains. The study also highlighted that accurate live/dead discrimination with IFC requires concentrations above 500,000 total particles/mL and that certain strains may require customized gating strategies, as default settings may not be optimal for all probiotic species [140]. Figure 6 presents a schematic comparison of the two flow-cytometric approaches.

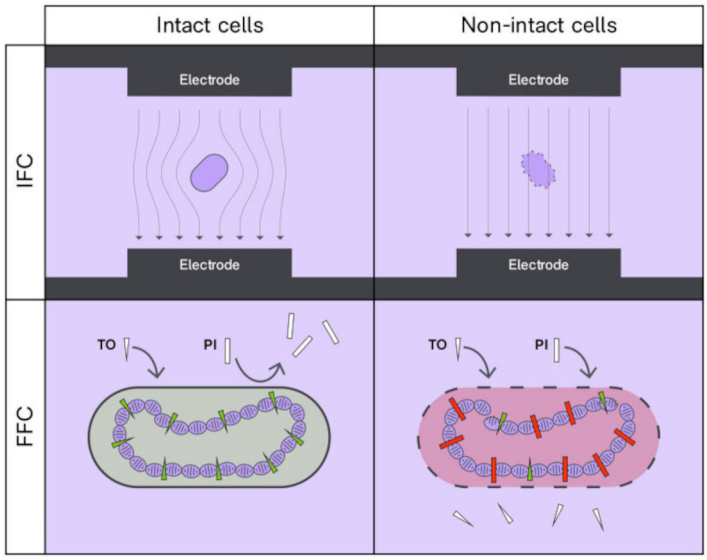

Figure 6. Assessment of membrane integrity by impedance (**up**) and fluorescence flow cytometry (**down**) [140].

8.2.2. Molecular Methods

Molecular methods based on nucleic acid detection enable highly specific and sensitive identification of bacteria at the species and even strain level, with high throughput. However, nucleic acid levels alone are not reliable indicators of cell viability: DNA can persist long after cell death, whereas RNA is unstable and can vary markedly with physiological state. Despite this limitation, molecular assays are extremely powerful when coupled to viability markers or viability dyes, enabling strain-selective detection of living cells [138].

Polymerase chain reaction (PCR)-based methods are widely applied for the detection and quantification of probiotic bacteria owing to their high specificity and sensitivity.

Real-time quantitative PCR (qPCR) employs fluorescent DNA-intercalating dyes (e.g., SYBR Green) or sequence-specific probes (e.g., TaqMan) to monitor amplification in real time. Target-specific primers flank the region of interest, and fluorescence increases with amplicon accumulation. Quantification is based on the quantification cycle (Cq), the point at which fluorescence exceeds background in the exponential phase, using a standard curve relating Cq to known copy numbers to estimate target abundance. For improved taxonomic resolution and reliability, single copy housekeeping genes (e.g., tuf, pheS) or markers from comparative genomics are preferred over 16S rRNA genes, which can exist in multiple copies and have high sequence similarity among closely related species. qPCR has practical constraints: it depends on a well-calibrated standard curve from purified DNA, can be affected by incomplete extraction and matrix inhibitors, and loses quantitative reliability at very low target abundance [138,143].

Digital PCR (dPCR) is an emerging third-generation PCR technology that enables absolute quantification without the need for standard curves. In dPCR, the mixture is partitioned into thousands of nanoliter microcompartments (e.g., droplets or chip wells), so that target molecules are distributed across partitions according to a Poisson distribution. After endpoint amplification, positive and negative partitions are counted and Poisson statistics are applied to correct for partition occupancy, including partitions containing more than one target molecule, and derive the absolute copy number. dPCR offers high precision, reduced susceptibility to inhibitors, and improved quantification of low-abundance targets, with good correlation to plate counts reported in several studies. Limitations include signal saturation at high template concentrations (requiring dilution), higher instrumentation costs, and workflow complexity.

A central limitation of both qPCR and dPCR is the inability to discriminate DNA from live versus dead cells, potentially inflating counts. To address this, assays can be coupled with membrane-impermeant photoactivatable dyes such as propidium monoazide (PMA) or ethidium monoazide (EMA). These dyes penetrate only cells with compromised membranes and, upon photoactivation, covalently modify DNA to block amplification. This viability PCR (vPCR) approach enables selective quantification of viable cells, though its reliability is highly protocol-dependent and influenced by dye concentration, exposure/activation conditions, matrix effects, and strain-specific membrane properties; rigorous controls and optimization are therefore essential [137,138,143].

8.2.3. Other Methods

Single-cell Raman spectroscopy (SCRS) is an emerging, culture-independent, non-invasive technique for rapid microbial characterization at single-cell resolution. In its conventional form, SCRS can also be label-free, as it relies on endogenous biochemical fingerprints of individual cells. By capturing these biochemical fingerprints, SCRS provides insights into metabolic state, viability, and taxonomic identity. When combined with stable-isotope probing, however, Raman-based workflows are no longer strictly label-free, since cells are exposed to isotopically labelled substrates or water to trace metabolic activity. A recent framework, called single-cell identification, viability, vitality, and sequencing (SCIVVS), combines SCRS with stable-isotope probing and single-cell genome sequencing for comprehensive probiotic analysis. In SCIVVS, cells are exposed to heavy isotopes (e.g., D_2O, ^{13}C, ^{15}N) that are incorporated by metabolically active cells; isotope incorporation produces characteristic Raman shifts (e.g., C–D bands at ~2040–2300 cm^{-1} for D_2O), enabling discrimination of live/active from inactive cells. SCRS-based workflows can also support species identification using reference spectral libraries and can link phenotypic traits to genomic information through downstream single-cell sequencing. Compared with PCR-based or flow-cytometric methods, SCRS offers a label-free biochemical fingerprint without nucleic-acid amplification, staining, or cultivation, whereas Raman stable-isotope probing provides a direct readout of metabolic activity through isotope incorporation. Nonetheless, while promising for probiotics (e.g., *Bifidobacterium* spp.), SCRS still requires further validation and optimization for large-scale, routine use, along with expansion and standardization of spectral databases to strengthen taxonomic resolution [143].

9. Conclusions

Drying is a critical determinant of probiotic product performance: it enables shelf-stable powders but can cause substantial viability losses if not carefully engineered. Evidence across technologies shows that survival reflects the interplay of formulation, process, and strain, rather than any single factor. Freeze drying remains one of the most widely used techniques for probiotic stabilization and is often reported as less detrimental to cell viability because of its relatively mild thermal conditions. However, it is associated with long cycle times, batch operation, and high energy demand. When formulation and process conditions are carefully optimized, alternative technologies, including conventional spray drying, spray freeze drying, and electrostatic spray drying, can achieve survival levels comparable to those reported for freeze drying in specific studies, while offering potential advantages in terms of processing time, continuity, scalability, or particle engineering. In addition to viability preservation, the choice of drying technology must also account for process robustness, scalability, product stability, and manufacturing costs, particularly in the development of pharmaceutical and nutraceutical probiotic formulations.

This review has outlined four complementary strategies to improve survival. (i) Stress adaptation (sublethal conditioning) can pre-activate protective responses and increase

robustness. (ii) Excipients, especially sugars, alone or combined with proteins or polysaccharides, can stabilize membranes and proteins via vitrification, water replacement, and interfacial protection; composite systems often outperform single agents but require careful ratio balancing. (iii) Encapsulation provides a physical barrier that moderates dehydration and oxidation while enabling tailored release. (iv) Process optimization, freezing profiles and annealing in FD temperature, residence time, and atomization windows in SD/SFD/ESD can substantially raise survival, although settings are inherently strain-specific and entail trade-offs among immediate viability, cycle time, residual moisture, and downstream functionality (e.g., rehydration, delivery). In this context, advanced analytical approaches and design-of-experiments methodologies may support the definition of strain-specific process windows while reducing empirical optimization efforts.

Robust viability assessment is also essential. Pairing culture-dependent counts with culture-independent tools (e.g., flow cytometry, molecular assays) reduces bias from sublethal injury and provides a more complete description of probiotic cell status. However, these methods should not be considered interchangeable, because they measure different endpoints, including culturability, membrane integrity, metabolic activity, or nucleic acid-based signals. The decision-making flowchart proposed in Figure 7 provides a practical guide for selecting viability assessment methods according to product type, production stage, analytical objective, and available equipment. The harmonization of analytical workflows and viability metrics will be important to improve comparability between studies and support future regulatory and quality-control frameworks for probiotic products.

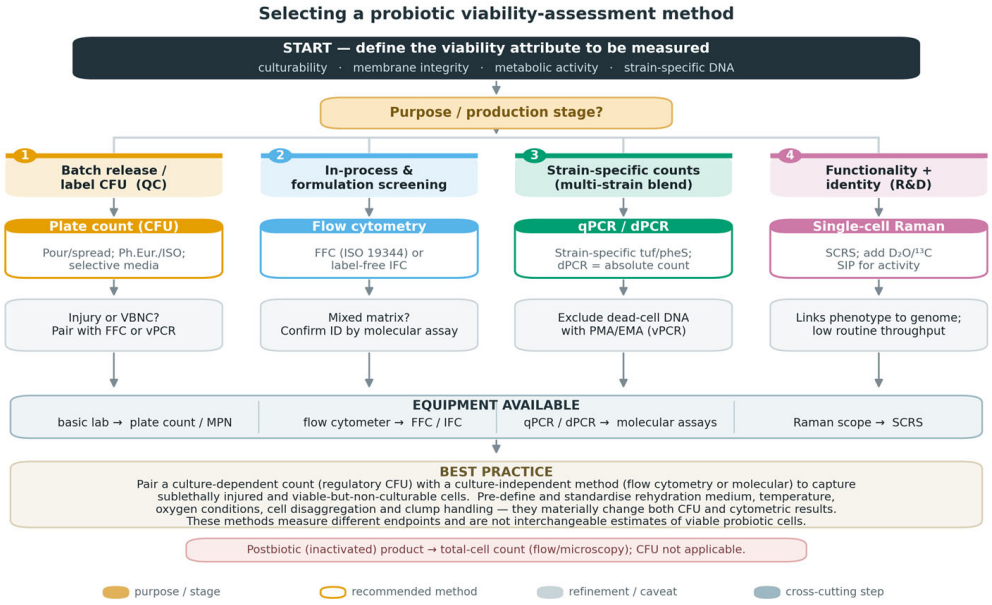

Figure 7. Decision-making flowchart for selecting probiotic viability assessment methods according to product type, production stage, analytical objective, and available equipment. The scheme highlights that culture-dependent, cytometric, molecular, metabolic, and spectroscopic approaches provide different and complementary information, and should therefore be selected or combined according to matrix complexity and the specific purpose of the analysis.

Post-drying storage stability and rehydration performance should also be considered as part of product design rather than as secondary endpoints. Storage temperature, moisture uptake, oxygen exposure, packaging permeability, glass-transition behaviour, and rehydration conditions can strongly influence both viable counts and strain-specific functional properties. Therefore, shelf-life assessment should integrate real-time and, where appropriate, accelerated studies, degradation kinetics, and overage definition to

ensure that the target viable dose and relevant functionality are maintained until the end of shelf life.

In sum, there is no universal recipe for preserving probiotic viability during drying. Effective solutions align strain-tailored formulations with process windows that safeguard structure while meeting industrial constraints. The strategies summarized here, adaptation, excipients, encapsulation, and targeted parameter optimization, provide a practical basis for designing next-generation probiotic powders with improved survival and functionality. Future advances in the field will likely rely on integrating process engineering, formulation science, and advanced viability analytics to develop scalable, stable, and clinically reliable probiotic products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr14152457/s1, Table S1: Excipients most frequently reported to improve probiotic survival during drying. The table summarizes the corresponding microbial species or strains, drying technique, drying conditions, protectant concentration and concentration basis, reported survival, comparator or control formulation, and reference. When multiple concentrations were reported within the same study, the best-performing concentration is underlined. FD: freeze drying; SD: spray drying; SFD: spray freeze drying; ESD: electrostatic spray drying.

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Abbreviations

The following abbreviations are used in this manuscript:

AFU	Active fluorescence units
a_w	Water activity
cFDA	5,6-carboxyfluoresceindiacetate
CFU	Colony forming units
Cq	Quantification cycle
dPCR	Digital polymerase chain reaction
EFSA	European Food Safety Authority
ESD	Electrostatic spray drying
FACS	Fluorescence-activated cell sorting
FAO	Food and Agricolture Organisation of the United Nations
FC	Flow cytometry
FD	Freeze Drying
FFC	Fluorescence flow cytometry
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
IFC	Impedance flow cytometry
LAB	Lactic acid bacteria
MRS	De Man, Rogosa, and Sharpe

n-AFU	Non-active fluorescence units
PCR	Polymerase chain reaction
PI	Propidium iodide
QbD	Quality by design
QPS	Qualified Presumption of Safety
qPCR	Real time quantitative polymerase chain reaction
ROS	Reactive oxygen species
SCIVVS	Single-cell identification, viability, vitality, and sequencing
SCRS	Single-Cell Raman Spectroscopy
SD	Spray Drying
SFD	Spray Freeze Drying
SFL	Spray freezing into liquid
SFV	Spray freezing into vapor
SFV/L	Spray freezing into vapor over liquid
TFU	Total fluorescence units
Tg	Glass transition temperature
TO	Thiazole Orange
VBNC	Viable but not culturable
v-PCR	Viability polymerase chain reaction
WHO	World Health Organisation

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