Probiotic raw-material dossier for *Bacillus*: Freeze-drying, cryoprotection, and compliance with pharmacopoeial vietnam standards

Ngo Nguyen Quynh Anh [1,2], Nguyen Thi Hai Yen [2], Dam Thanh Xuan[3*]

[1]Hai Duong Central College of Pharmacy
[2] Hanoi University of Pharmacy

Summary

This study investigated the impact of cryoprotectants on the quality and stability of freeze-dried powders of *Bacillus subtilis* M70 and *Bacillus clausii* M31, in compliance with the Vietnamese Pharmacopoeia V standards. Biomass was cultured, harvested, and suspended in protective media containing single (10% w/w) or binary (5% + 5% w/w) cryoprotectants. Samples were pre-frozen at -80 °C and lyophilized using a programmed cycle (-50 °C primary drying; 0.055 bar). Quality attributes, including macroscopic properties, moisture content, morphology, and viable cell count (CFU/g), were assessed immediately and after six months of storage at 4-6 °C. Among single agents, 10% mannitol produced the most favorable results, yielding dry, porous powders with consistent flowability and maintaining viability above 10^{10} CFU/g for both strains. Specifically, *B. subtilis* retained 10.27 log CFU/g after six months. In contrast, the binary combination of 5% trehalose + 5% maltodextrin exhibited a synergistic and strain-specific effect on *B. clausii*, ensuring the highest survival rate (10.54 log CFU/g) and superior powder morphology. All optimized formulations produced stable powders with low residual moisture and porous structures, meeting pharmacopoeial requirements while preserving probiotic viability above the threshold of 10^{10} CFU/g. Statistical analysis confirmed significant differences ($p < 0.05$) between optimized formulations and controls. These findings highlight the importance of tailored cryoprotectant strategies for spore-forming probiotics. Mannitol was identified as the optimal protectant for *B. subtilis*, while trehalose–maltodextrin combinations offered superior protection for *B. clausii*. The results provide a practical basis for the development of stable probiotic raw materials suitable for pharmaceutical and functional food applications.

Keywords: Freeze-drying, cryoprotectant, *Bacillus subtilis*, *Bacillus clausii.*

I. BACKGROUND

Probiotics, particularly *Bacillus clausii* and *Bacillus subtilis,* play a pivotal role in promoting human and animal health through modulation of the gut microbiota [1]. These two species are distinguished by their ability to form spores, enabling them to withstand harsh conditions such as elevated temperature, low pH, and other processing- or digestion-related stresses [2]. Their applications continue to expand across pharmaceuticals, dietary supplements, and livestock/aquaculture production systems, especially in developing countries such as Vietnam [3].

Freeze-drying, while widely regarded as an effective means to stabilize viable microorganisms, can inflict dehydration-induced membrane damage that substantially reduces post-process viability [4]. To mitigate this, the use of cryoprotectants, such as mannitol, trehalose, or glycerin, has become essential, with protective efficacy being highly strain-dependent [5].

***Received**: 21/10/2025, **Accepted**: 27/11/2025*
***Correspondence to**: xuandt@gmail.com*
Hanoi University of Pharmacy

In Vietnam, the probiotic industry remains largely reliant on imported freeze-dried biomass, and many domestically produced products still show limitations in cell density, stability, and shelf life [6]. This dependence on foreign raw materials increases production costs, reduces competitiveness of local enterprises, and poses risks to supply chain resilience. Developing robust, high-quality probiotic raw materials within the country is therefore strategically important not only to reduce dependency on imports, but also to secure a sustainable foundation for the pharmaceutical and functional food industries [7, 23].

Furthermore, the rational use of spore-forming probiotics such as *B. clausii* has been linked to reduced recurrence of infections and mitigation of antibiotic-associated dysbiosis, thereby contributing to the global effort against antimicrobial resistance (AMR) [2, 5, 6]. Incorporating domestically produced, stable probiotic raw materials into therapeutic regimens could help reduce the overuse of antibiotics, improve patient outcomes, and support public health strategies on AMR containment [24].

International studies have demonstrated that combinations of trehalose, sucrose, glycerin, and skim milk can markedly improve survival in *Lactobacillus* spp. [8]. However, each genus and species exhibits distinct requirements regarding excipient selection, loading, and blending ratios, parameters that critically influence outcomes [9, 21].

These insights underscore the urgent need for Vietnam to advance freeze-drying technologies for *B. clausii* and *B. subtilis*, both of which hold significant potential in pharmaceutical and husbandry applications.

Accordingly, this study aims to identify effective cryoprotectant systems that ensure freeze-dried *B. clausii* and *B. subtilis* meet the specifications of the Vietnamese Pharmacopoeia V while maintaining high viability over time.

2. MATERIALS AND METHODS

2.1. Microbial strains

Bacillus subtilis M70 and *Bacillus clausii* M31 (previously isolated and identified) were preserved at the Center for Pharmaceutical Research and Business, Hai Duong Central College of Pharmacy [11, 13]. Cryoprotectants included mannitol (*China*), maltodextrin (*USA*), skim milk (*India*), trehalose (*USA*), glycerin (*Malaysia*), sucrose (*Vietnam*), and lactose (*India*) at 5 - 10% (w/w), all meeting pharmaceutical-grade specifications.

2.2. Experimental methods

2.2.1. Biomass preparation Bacillus spp.

B. subtilis M70 and *B. clausii* M31 were cultivated in liquid LB medium (*Luria–Bertani Broth, Himedia)* at 37°C for 24h. Cells were harvested by centrifugation (4,000 rpm, 15 min; Hettich Eba 20, Germany) and washed twice with sterile distilled water [10]. The biomass was then suspended in protective media containing excipients (either single 10% w/w or binary 5% + 5% w/w), transferred into sterile test tubes, sterilized (115°C, 20 min), and subsequently poured into sterile petri dishes.

2.2.2. Freeze-drying

Prepared samples were pre-frozen at –80 °C for 24 h. Lyophilization was performed in a freeze-dryer (Christ Alpha, Germany) with the following cycle: primary drying at -50 °C to -10 °C (0.055 bar) for 24 h, followed by secondary drying at 20 °C (0.01 bar) for 6 h to ensure low residual moisture.

2.2.3. Evaluation of lyophilized powder

Macroscopic attributes were assessed at 25 ± 2°C and 40-50% RH using a stainless-steel spatula and clean tray. Dryness and fluffiness were scored on a 0-10 scale based on adherence to the spatula, agglomerate size (mm), and flowability. Each sample was evaluated 2-3 times and averaged [10]. Moisture content was determined using a moisture analyzer (Ohaus, USA) at 105°C until constant weight. Viability was determined by serial dilution plating on LB agar (37 °C, 24-48 h) and expressed as CFU/g [11]. Bacterial identity was confirmed qualitatively [3], while microbial contamination was assessed according to the microbial limit test [8]. Powder microstructure was examined by scanning electron microscopy (*SEM;* Nova Nano SEM *450, USA*) at 2,500× and 5,000×

magnification. Stability was evaluated after 6 months of storage at 4-6 °C.

2.2.4. Data acquisition and analysis

All data were processed using Microsoft Excel 2016 and expressed as mean ± standard deviation (n = 3) [13].

III. RESULT

3.1. Selection of cryoprotectants and evaluation of physical attributes

Both single (10% w/w) and binary (5% + 5% w/w) cryoprotectant systems were screened to evaluate the physical state and residual moisture of freeze-dried powders of *Bacillus subtilis* and *Bacillus clausii*.

Across the 23 formulations assessed, compositions containing glycerin at ≥ 5% in combination with lactose, maltodextrin, or sucrose consistently produced foamed, syrupy, or tacky masses after lyophilization. In contrast, systems based on mannitol, skim milk, and trehalose showed more favorable cake formation. Representative results for *B. subtilis* are summarized in Table 1.

Table 1. Physical evaluation of cryo/lyoprotectant formulations for freeze-drying *Bacillus subtilis M70*

Formu -lation	Composition	Physical appearance	Attributes
CT1	Sucrose 10%	Caked, brownish	70% adhesion, >5 mm, non-fluffy
CT2	Lactose 10%	Caked, yellow	20% adhesion, 3–5 mm, poor flow
CT3	Skim milk 10%	Caked, brown	5% adhesion, no lumps, free-flowing
CT4	Maltodextrin 10%	Caked, off-white	10% adhesion, <3 mm, good flow
CT5	Mannitol 10%	Caked, white	5% adhesion, no lumps, free-flowing
CT6	Trehalose 10%	Caked, brownish	20% adhesion, 3–5 mm, poor flow
CT7	Glycerin 10%	Syrupy, translucent	100% adhesion, flowing, non-fluffy
CT8	Lactose 5% + Sucrose 5%	Caked, yellow	10% adhesion, <3 mm, good flow
CT9	Skim milk 5% + Glycerin 5%	Slight caking, yellow-brown	10% adhesion, <3 mm, good flow
CT10	Mannitol 5% + Glycerin 5%	Caked, white	5% adhesion, no lumps, free-flowing
CT11	Trehalose 5% + Maltodextrin 5%	Caked, yellow-brown	5% adhesion, no lumps, free-flowing
CT12	Lactose 5% + Glycerin 5%	Foamy, tacky, dark brown	70% adhesion, >5 mm, non-fluffy

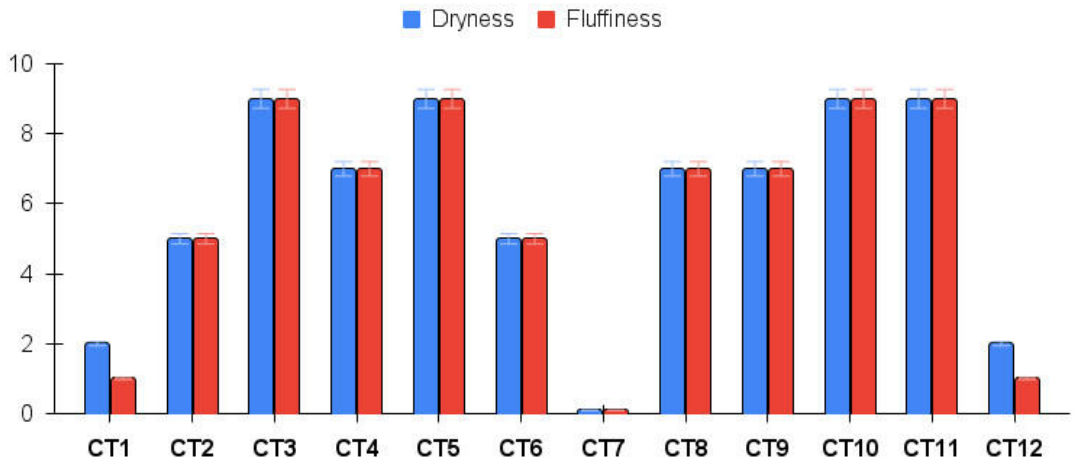


Figure 1. Dryness and fluffiness evaluation of powder formulations of *B.subtilis* M70

Notes: Dryness (0 - 10): 10 = non-tacky (0% adhesion); 7 - 9 = slightly tacky (< 10%); 4 - 6 = moderately tacky (10 - 30%); 0 - 3 = very tacky/syrupy (> 50%). Fluffiness (0 - 10): 10 = free-flowing (< 3 mm); 7 - 9 = fluffy, agglomerates < 3 mm; 4 - 6 = limited fluffiness, 3 - 5 mm; 0 - 3 = non-fluffy, > 5 mm/syrupy.

Overall, the physical evaluation revealed marked differences between single-component and multi-component systems (*Fig. 1*). CT3 (10% skim milk), CT5 (10% mannitol), CT10 (5% mannitol + 5% glycerin), and CT11 (5% trehalose + 5% maltodextrin) achieved the highest mean scores (9.0), characterized by optimal dryness and fluffiness (non-tacky, free-flowing, no agglomeration). These observations suggest that skim milk and mannitol, and, among binary systems, trehalose-maltodextrin, are promising cryoprotectants for maintaining a dry, porous cake conducive to long-term storage and downstream handling of *B. subtilis*.

By contrast, CT1 (10% sucrose) and CT12 (5% lactose + 5% glycerin) scored poorly (1.5), driven by pronounced tackiness (> 70% adhesion) and large agglomerates (> 5mm), indicating that sucrose alone and lactose - glycerin at the tested levels may be suboptimal. CT7 (10% glycerin) performed worst (0.0), exhibiting a syrupy, fully adhesive mass-consistent with the strong hygroscopicity of glycerin. Intermediate performers (CT2, CT4, CT6, CT8, CT9; mean 5.0 - 7.0) were acceptable but would likely benefit from further optimization of concentration or co-formulation to reduce tackiness (10 - 20% adhesion, 3 - 5mm agglomerates). Taken together, the data support mannitol and trehalose-containing systems, particularly with maltodextrin or dairy solids, as leading candidates for subsequent viability and stability studies.

Table 2. Physical evaluation of cryoprotectant formulations for freeze-drying *B.clausii* M31

Formu -lation	Composition	Physical appearance	Attributes
CT13	Sucrose 10%	Slight caking, yellowish-white	10% adhesion, <3 mm, good flow
CT14	Lactose 10%	Caked, yellow	20% adhesion, 3–5 mm, poor flow
CT15	Skim milk 10%	Caked, yellowish-white	5% adhesion, no lumps, free-flowing
CT16	Maltodextrin 10%	Caked, yellowish-white	10% adhesion, <3 mm, good flow
CT17	Mannitol 10%	Caked, white	5% adhesion, no lumps, free-flowing

Formu -lation	Composition	Physical appearance	Attributes
CT18	Trehalose 10%	Caked, yellowish-white	10% adhesion, <3 mm, good flow
CT19	Lactose 5% + Sucrose 5%	Caked, yellow	10% adhesion, <3 mm, good flow
CT20	Skim milk 5% + Glycerin 5%	Caked, yellow	10% adhesion, <3 mm, good flow
CT21	Mannitol 5% + Glycerin 5%	Porous, yellow-brown	20% adhesion, 3–5 mm, poor flow
CT22	Trehalose 5% + Maltodextrin 5%	Caked, white	5% adhesion, no lumps, free-flowing
CT23	Lactose 5% + Glycerin 5%	Syrupy/tacky, dark brown	70% adhesion, >5 mm, non-fluffy

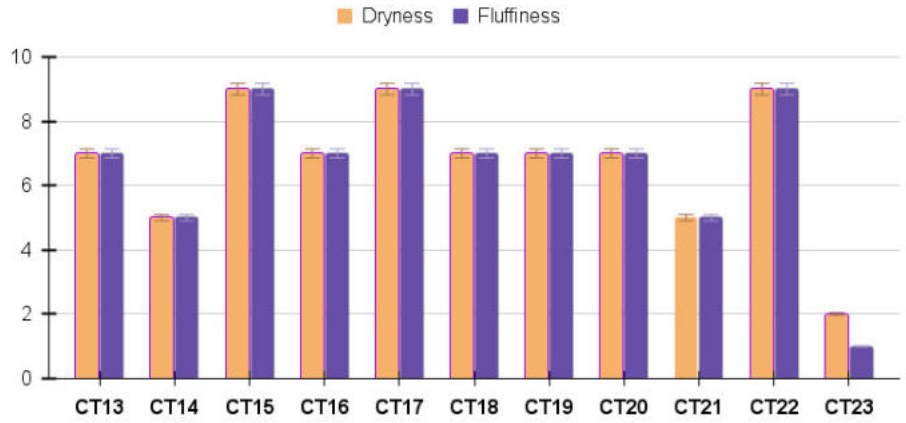


Figure 2. Dryness and fluffiness evaluation of powder formulations of *Bacillus clausii* M31

Notes: Dryness (0 - 10): 10 = non-tacky (0% adhesion); 7 - 9 = slightly tacky (< 10%); 4 - 6 = moderately tacky (10–30%); 0 - 3 = very tacky/syrupy (>50%). Fluffiness (0 - 10): 10 = free-flowing (< 3mm); 7 - 9 = fluffy, agglomerates < 3mm; 4 - 6 = limited fluffiness, 3 - 5mm; 0 - 3 = non-fluffy, >5 mm/syrupy.

The physical evaluation of cryoprotectant systems for freeze-drying *B. clausii* revealed notable differences in powder characteristics and overall quality (Fig. 2). CT15 (10% skim milk), CT17 (10% mannitol), and CT22 (5% trehalose + 5% maltodextrin) achieved the highest mean scores (9.0), reflecting favorable physical profiles with optimal dryness and fluffiness (5% adhesion, no agglomeration, free-flowing). These findings suggest that skim milk, mannitol, and the trehalose-maltodextrin combination are effective cryoprotectants for maintaining a stable, porous cake suitable for long-term storage and practical use of *B. clausii*. In contrast, CT23 (lactose 5% + glycerin 5%) yielded the lowest mean score (1.5) due to pronounced tackiness (70% adhesion, > 5mm agglomerates) and poor fluffiness, indicating that the lactose-glycerin pairing is suboptimal, likely owing to hygroscopicity. CT21 (mannitol 5% + glycerin 5%) had an intermediate mean score (5.0), with moderate tackiness (20% adhesion, 3–5 mm) and poor flow, suggesting only average performance. The remaining formulations (CT13, CT14, CT16, CT18, CT19, CT20) scored 5.0 - 7.0, indicating acceptable attributes (≈10% adhesion, < 3 mm, good flow) that may be further improved by adjusting levels or combinations of cryoprotectants. Overall, these data support selecting cryoprotectants based on both physical behavior and anticipated protective capacity in subsequent viability assays, with skim milk, mannitol, and trehalose-maltodextrin emerging as leading candidates.

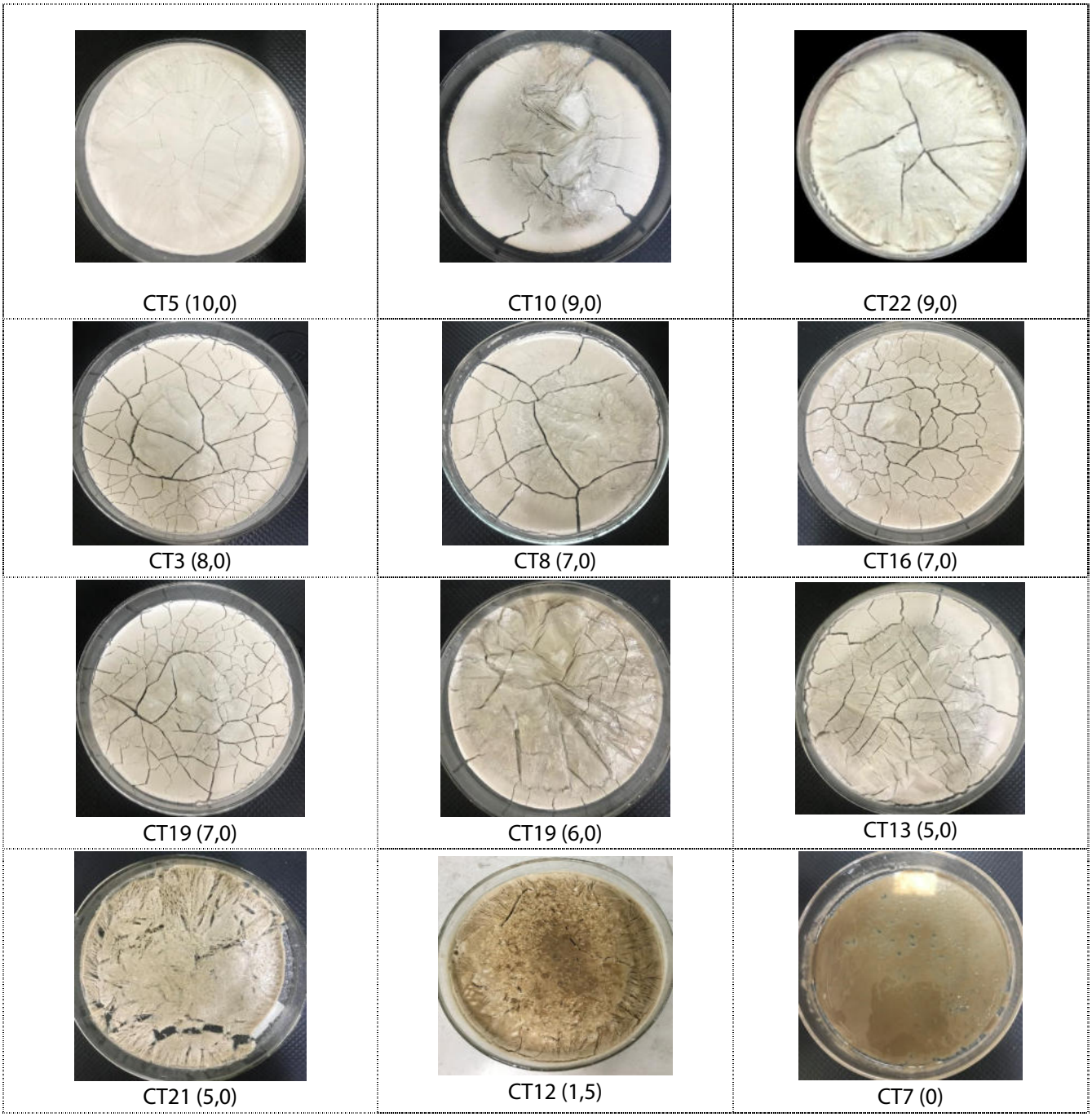


Figure 3. Freeze-dried powder samples showing variations in physical characteristics according to organoleptic evaluation

In *Figure 3*, the freeze-dried powders from CT5 (10% mannitol) and CT3 (10% skim milk) attained high organoleptic scores (10.0 and 7.0, respectively), presenting dry, free-flowing cakes (0-5% adhesion, < 3mm). These attributes may confer superior bacterial protection relative to poorer textures. By contrast, CT7 (10% glycerin) scored 0.0, appearing syrupy (100% adhesion) and non-fluffy, reinforcing that glycerin is unsuitable in this context. CT13 (10% sucrose) scored 5.0, with moderate tackiness (20% adhesion, 3-5 mm) and average overall performance warranting optimization.

3.2. Quality evaluation of freeze-dried powders and establishment of an in-house specification for probiotic lyophilizates

A comprehensive quality assessment was conducted on freeze-dried powders after shortlisting several cryo/lyoprotectant formulations, with the aim of drafting an in-house quality specification aligned with the Vietnamese Pharmacopoeia V requirements.

a. Organoleptic properties

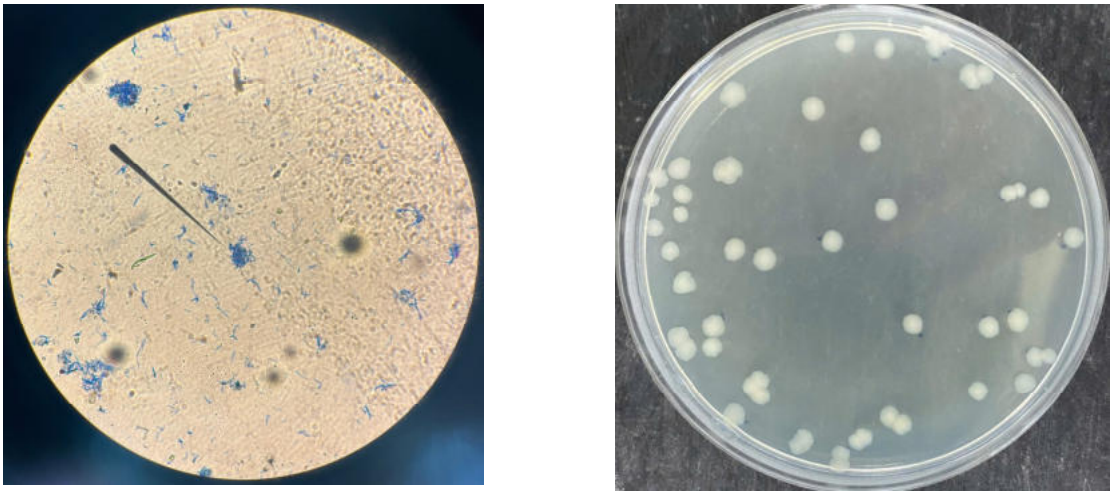
Freeze-dried powders meeting acceptance criteria exhibited a dry, free-flowing, fine, and homogeneous texture, ivory-white in color, without off-odors and without caking/lumping (*Fig. 4a*).

b. Bacterial identification

To confirm identity and purity of *B. clausii* in the bulk, qualitative tests were undertaken in *Fig. 4b*

- Gram staining: Bacteria subcultured from the freeze-dried powder stained Gram-positive (blue-purple), with rod-shaped morphology occurring singly or in short chains, features consistent with the genus *Bacillus*.

- Colony morphology: Colonies were circular, white to gray-white, with diameters reported at 3-6 µm, and with entire margins or fine serrations.



a. After Gram staining b. Colony morphology

Figure 4. Microscopic observations and colony morphology on LB agar plates.

Biochemical profiling to characterize metabolic traits is summarized in *Table 3*.

Table 3. Biochemical test results of the strains

Parameter	***B. subtilis* M70**	***B. clausii* M31**
1. Cell morphology	Rod, *Bacillus*	Rod, *Bacillus*
2. Gram stain	Gram-positive	Gram-positive
3. Catalase	(+)	(+)
4. Oxidase	(+)	(+)
5. Spore formation	(+)	(+)
6. Voges-Proskauer	(+)	(+)
7. Nitrate reduction	(+)	(–)
8. Citrate utilization	(+)	(+)
9. Motility	(+)	(+)

Notes: (+) positive; (–) negative

The biochemical results in *Table 3* for strain M70, M31 were consistent with reference profiles for *B. subtilis* and *B. clausii* [3, 14].

c. Viable cell counts

Viable counts (CFU/g) were determined immediately after lyophilization and after 6 months of storage. Both strains retained high viable counts in the best-performing formulations. Detailed data are presented in Fig 5.

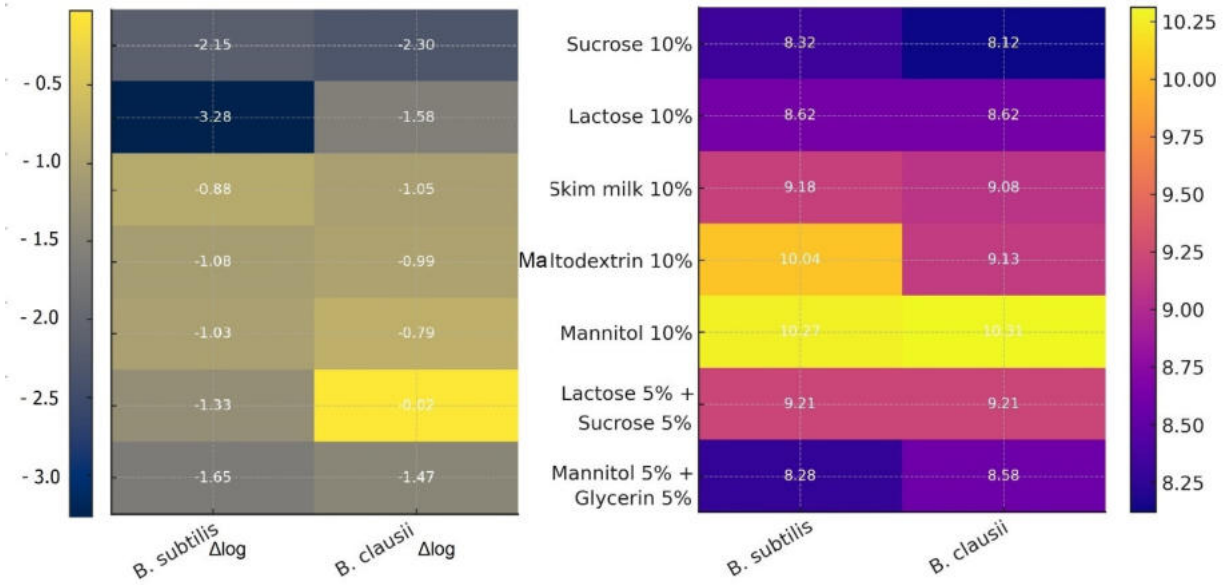


Figure 5. Comparative survival of *B. subtilis* and *B. clausii* (log CFU/g)

The survival data in *Table 4* highlight clear, formulation-dependent differences in post-lyophilization viability and 6-month stability for *Bacillus subtilis* and *Bacillus clausii*. For *B. subtilis*, 10% mannitol (CT5) and 10% maltodextrin (CT4) were the top single-excipient performers, sustaining ≥10.0 log CFU/g after 6 months. Statistical analysis showed no significant difference between CT4 and CT5 ($p>0.05$), but both were superior to CT1 and CT2 ($p<0.05$). Skim milk (CT3) was moderately protective, whereas sucrose (CT1) and lactose (CT2) alone were least protective. For *B. clausii*, the binary system trehalose 5% + maltodextrin 5% (CT23) and mannitol 10% (CT17) were superior followed by maltodextrin (CT16) and skim milk (CT15) with ~1-log loss. Formulations with glycerin (CT10, CT22) showed greater losses. Notably, the lactose + sucrose pair (CT20) exhibited minimal decline but its absolute counts were lower at baseline, illustrating the distinction between "retention" and "end-of-shelf-life absolute CFU".

Expressed as log differences over 6 months, *B. subtilis* showed Δlog ≈ -1.03 (CT5), -1.08 (CT4), -0.88 (CT3), compared with -2.15 (CT1) and -3.28 (CT2). For *B. clausii*, Δlog ≈ -0.48 (CT23), -0.79 (CT17), -0.99 (CT16), -1.05 (CT15), versus -2.30 (CT13) and -1.58 (CT14). In relative terms, these translate to approximate viability retentions of ~9 -13% for the best *B. subtilis* systems and ~16 - 33% for the best *B. clausii* systems over 6 months at 4 - 6°C. While single-digit retention may appear modest, the high starting titers (≥10^{11} CFU/g in the best formulations) ensure that absolute counts remain ≥10^{10} CFU/g at 6 months, often the more critical parameter for label claims and downstream processing.

Polyols (mannitol) and higher-molecular-weight saccharides/polymers (maltodextrin; trehalose-containing blends) consistently outperformed

simple disaccharides (sucrose, lactose) as single agents. Mannitol likely contributed a rigid, crystalline scaffold that limited structural collapse and meltback during freeze-drying, while maltodextrin and trehalose, single or in combination, supported glass formation and water replacement, reducing dehydration-induced damage. Glycerin-containing systems underperformed at the tested levels, consistent with its hygroscopicity and tendency to yield tacky matrices that are less favorable for long-term stability in dry powders.

The trehalose + maltodextrin system (CT23) was distinctly better for *B. clausii* than for *B. subtilis* (10.54 and 9.04 log CFU/g at 6 months in their respective CT11/CT23 positions across tables), suggesting strain-specific interactions with the spore coat and matrix glass-transition behavior. Conversely, *B. subtilis* appeared to benefit more from mannitol (CT5) and maltodextrin (CT4) singles, indicating that a single rigid scaffold or a moderately glassy matrix can suffice when starting loads are high and the spore is intrinsically robust.

CT20 (*B. clausii*, lactose + sucrose) is a cautionary example: despite excellent percent retention ($\Delta\log \approx -0.02$), its absolute CFU remained around 9.2 at both time points, below the $\geq 10^{10}$ target typically sought for raw material destined for compression, coating, and distribution. From a registration and product-design standpoint, end-of-shelf-life absolute CFU per unit is the binding constraint; thus, the formulations with higher initial titers and acceptable losses (CT23, CT17) are preferable to a "stable but low" option (CT20).

The relatively modest 6-month losses (~0.5–1.1 log) for the best systems imply a well-tuned freeze-drying cycle (e.g., adequate primary drying, restrained product temperature, secondary drying sufficient to lower residual moisture). This process control likely enabled mannitol to crystallize into a stable form and allowed trehalose/maltodextrin matrices to vitrify effectively, both conditions that are known to support long-term stability. Results were generated at 4-6°C; extrapolation to room temperature or ICH long-term/accelerated conditions requires further study. The dataset focuses on two spore-forming strains and a defined set of excipient levels; optimization across a broader design space such as mannitol/trehalose ratios, alternative dextrins, pre-conditioning osmolarity, annealing steps that may yield further gains. Finally, translation into finished dosage forms (direct compression tablets, pellets, or enteric-coated tablets…) should include stress mapping (compaction, heat, solvent exposure) and dissolution in SGF/SIF with CFU recovery.

Building on the above results and process recommendations, a pragmatic development pathway is to first select the most suitable excipient systems (10% mannitol for *Bacillus subtilis*; 5% trehalose + 5% maltodextrin or 10% mannitol for *Bacillus clausii*), then lock down the freeze-drying cycle within a QbD framework, and treat packaging-storage as a first-order design variable. This cautious, stepwise approach is intended to preserve end-of-shelf-life CFU: clearly defined CMAs/CPPs maintain viability through freeze-drying, whereas high-barrier moisture/oxygen packaging and cold-chain storage at 4-6°C minimize rehydration-driven loss. The findings should be considered preliminary; additional work is required, including scale-up verification, ICH stability matrices (25°C/60% RH; 30°C/65% RH; 40°C/75% RH), and quantification of CFU attrition across downstream unit operations (blending, compression, enteric coating) alongside SGF→SIF dissolution with CFU recovery. Complementary evaluations of cost-effectiveness, lot-to-lot variability, and long-term batch stability will further refine specifications and support the registration dossier. Overall, an integrated formulation-process-packaging strategy provides a feasible foundation to translate these data into stable, registration-ready probiotic products.

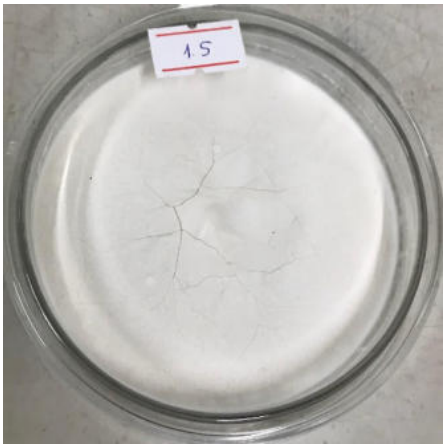
For freeze-dried *Bacillus* probiotics intended for high label claims and downstream processing, mannitol 10% (for *B. subtilis*) and trehalose 5% + maltodextrin 5% or mannitol 10% (for *B. clausii*) provide the best balance of cake quality, immediate post-process titers, and 6-month stability at 4–6°C. These findings translate directly into formulation

selection, process control, packaging strategy, and specification setting for development and registration.

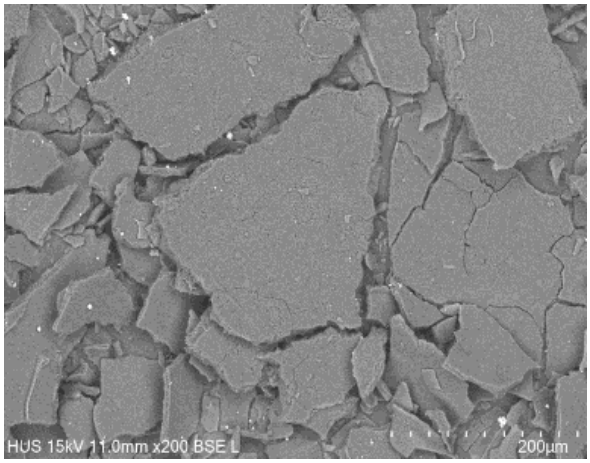
d. Microbial limits

Freeze-dried bulk materials were tested against the microbial limit specifications of the Vietnamese Pharmacopoeia V. *E. coli*, *Salmonella* spp., *S. aureus*, and bile-tolerant Gram-negative bacteria were not detected in 1g of powder, satisfying purity and safety requirements per the Pharmacopoeia.

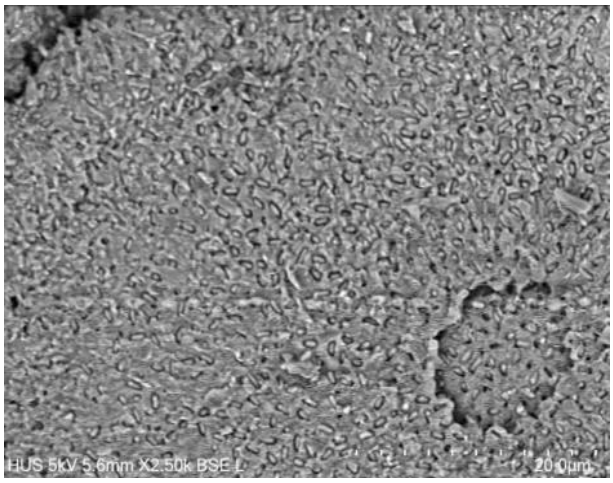
g. Microstructural evaluation and encapsulation efficiency



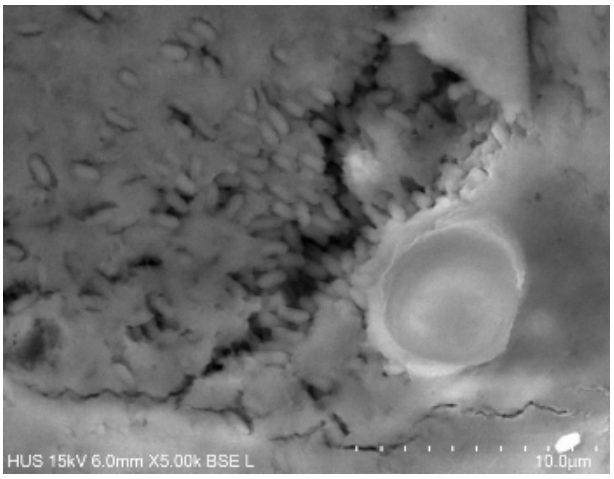
a. freeze-dryied powder



b. 200. X



c. 2.500X



d. 5.000X

Figure 6. Scanning electron micrographs of Freeze-dried powder (CT5)

Powder morphology and microstructure. Macroscopic inspection indicated that the lyophilized product exhibited a highly desirable texture. The material formed a robust, intact cake with a flat surface and a uniformly smooth, ivory-white appearance. These features suggest that the lyophilization cycle was well controlled, with no evidence of structural collapse or meltback, early indicators of an effective cryoprotectant formulation.

Porous network architecture (*Fig 6a, 6b*). At low magnification, the images clearly revealed a multilayered, tightly interconnected porous network. Such architecture is characteristic of uniform water sublimation, leaving voids that constitute a rigid "scaffold," thereby helping the product retain its original shape and volume.

Spore distribution (*Fig 6c*). At 2,500× magnification, rod-shaped *Bacillus* spores were

readily observed, displaying uniform size and intact morphology. Importantly, spores appeared evenly distributed throughout the excipient matrix, indicating that mixing and pre-freezing were effective, with no apparent clumping or microbial aggregation.

Micro-encapsulation efficiency (Fig 6d). At the highest magnification (5,000×), the micrographs showed dense populations of spores seemingly "embedded" in or firmly adhered to excipient particle surfaces. This finding suggests that the excipients formed an effective micro-scale coating, immobilizing and protecting individual spores from mechanical stress such as ice-crystal damage) and chemical stress (dehydration, oxidation) during freeze-drying and storage. Such features plausibly underlie the high post-process viability documented.

IV. DISCUSSION

The efficacy of cryo/lyoprotectants is fundamentally linked to their ability to attenuate ice-crystal

damage and dehydration stress during freeze-drying [4]. In the present study, 10% mannitol consistently demonstrated superior protective efficacy for both *B. subtilis* and *B.clausii*, with viable counts exceeding 10^{10} CFU/g after six months of storage. Although earlier reports have cautioned against mannitol due to potential crystallization into hemihydrates or polymorphic transitions under stress [14, 16], these concerns have primarily been observed in more labile systems such as proteins or vegetative Lactobacillus cells. By contrast, *Bacillus* spores possess inherent structural resilience [2], and under tightly controlled lyophilization conditions (-50°C primary drying; 4-6°C storage), mannitol appeared to remain in a stable crystalline state, functioning effectively as a structural scaffold without destabilizing effects. This observation resonates with the findings of Kim et al (1998), who emphasized that the physical state of mannitol is highly dependent on concentration and process parameters [15].

A second important insight concerns the strain-specific synergy of excipient combination**s**. The formulation of 5% trehalose + 5% maltodextrin provided excellent stabilization for B. clausii (10.54 log CFU/g) but offered comparatively lower protection for B. subtilis (9.04 log CFU/g). This suggests that the interaction of excipients with the spore coat is not universal, but instead reflects subtle physicochemical compatibilities. Mechanistically, trehalose is known to form an amorphous glassy matrix that immobilizes intracellular structures and reduces mechanical stress [16, 22] which may be particularly effective for *B. clausii* spores [30]. Such insights provide a mechanistic basis for tailoring excipient selection to specific microbial targets rather than applying a "one-size-fits-all" approach.

In global context, these results position Bacillus spores as particularly robust probiotic candidates. Studies in Europe and East Asia have often required multi-component, complex formulations to preserve *Lactobacillus* viability during freeze-drying [7, 8, 11]. For example, Hom et al (2018) reported high survival of Lactobacillus only with sophisticated mixtures of sugars, polyols, and polymers [8], whereas our study achieved superior outcomes with simpler binary formulations. Compared with domestic work in Vietnam, such as the early study by Le Ngoc Tran (2008) on lactic acid bacteria using basic excipients [18, 20] our findings suggest that mannitol and trehalose represent more effective candidates, especially for long-term stability and industrial scalability.

From a national biotechnology perspective, these findings have direct implications for establishing raw material standards in Vietnam. Probiotic production remains constrained by variability in input quality, and the absence of harmonized excipient standards limits industrial reproducibility. By demonstrating that 10% mannitol alone, or in combination with trehalose/maltodextrin, can reliably yield spore preparations with > 10^{10} CFU/g over extended storage, this study contributes an evidence-based

foundation for defining pharmacopoeial-grade stabilizers in probiotic manufacturing. Aligning these standards with the Vietnamese Pharmacopoeia [8] will not only support domestic consistency but also facilitate international comparability, enabling Vietnam's probiotic sector to meet the stringent quality benchmarks already established in regions such as the EU and Japan, where regulatory frameworks tightly couple excipient composition with product stability and efficacy [6, 28, 30].

Finally, rather than prescribing a fixed set of formulations, it may be more appropriate to establish a flexible framework that recognizes both the robustness of Bacillus spores and the variability of industrial conditions. Such a framework would allow manufacturers to innovate within scientifically supported boundaries, while gradually aligning with international expectations for probiotic quality and safety.

In this way, the current work may serve as one step toward a longer-term vision: contributing practical data to a shared national effort of standard-setting in probiotic biotechnology by codifying excipient standards into national guidelines, and reducing dependence on imported stabilizer formulations. Such an approach mirrors international best practices while ensuring contextual adaptation to local manufacturing capacity and regulatory landscapes. In doing so, Vietnam can position itself not only as a consumer but as a regional producer of high-quality probiotic raw materials, contributing to global health solutions through scalable, affordable, and scientifically validated biotechnologies.

While this study established effective formulations for cold storage (4–6 °C), we acknowledge limitations regarding stress testing. Specifically, stability under accelerated conditions (ICH zone IVb: 30°C/75% RH or 40°C/75% RH) and glass transition temperature (Tg) measurements were not included in this scope. Although Tg is a critical parameter for understanding long-term stability mechanisms, the high viability retention (>10^{10} CFU/g) suggests that the optimized cycle successfully minimized molecular mobility at the storage temperature utilized. Future studies will focus on detailed thermal analysis (DSC) and stress testing (heat shock, moisture tolerance) to transition these raw materials into finished dosage forms.

From an industrial perspective, the recommended formulations (10% mannitol for *B. subtilis* and trehalose-maltodextrin for *B. clausii*) utilize readily available, cost-effective, and pharmacopoeia-compliant excipients. This provides a feasible foundation for domestic manufacturers to produce high-quality probiotic raw materials, reducing reliance on imports and ensuring supply chain autonomy.

V. CONCLUSION

This study evaluated cryoprotectant formulations for lyophilizing two probiotic strains, *B. subtilis* M31 and *B. clausii* M70. Mannitol 10% was identified as the optimal single-excipient system for *B. subtilis*, maintaining 10.27 log CFU/g after 6 months. For *B. clausii*, the 5% trehalose + 5% maltodextrin combination provided superior protection, sustaining the highest viable counts (10.54 log CFU/g) at 6 months. In all optimal formulations, the resulting powders were dry and free-flowing and consistently maintained viable counts above 10^{10} CFU/g. These results support further exploration of lyophilization conditions and complementary excipients to enhance long-term stability and contribute to the advancement of biopharmaceutical manufacturing capabilities in Vietnam.

REFERENCES

1. Elisashvili V, Kachlishvili E, Chikindas ML (2019) *Recent advances in the physiology of spore formation for Bacillus probiotic production*. Probiotics Antimicrob Proteins 11(3): 731-747.
2. Ghelardi E, Abreu Y, Abreu AT et al (2022) *Current Progress and Future Perspectives on the Use of Bacillus clausii*. Microorganisms 10(6): 1246.

3. Li X, Che L, Wu Y et al (2024) *An effective strategy for improving the freeze-drying survival rate of Lactobacillus curvatus and its potential protective mechanism*. Food Bioscience 58: 103794.
4. Chen B, Wang X, Li P et al (2023) *Exploring the protective effects of freeze-dried Lactobacillus rhamnosus under optimized cryoprotectants formulation*. LWT 173: 114295.
5. Hill C, Guarner F, Reid G et al (2014) *The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic*. Nat Rev Gastroenterol Hepatol 11: 506–514.
6. Wang G, Yu X, Lu Z et al (2019) *Optimal combination of multiple cryoprotectants and freezing-thawing conditions for high lactobacilli survival rate during freezing and frozen storage*. LWT 99: 217-223.
7. Bộ Y tế (2017) *Dược điển Việt Nam V*. Nhà xuất bản Y học, Hà Nội, tập 2, PL 13.6.
8. Nguyễn Thị Hường, Vũ Thị Thu Hằng, Nguyễn Văn Thắng và cộng sự (2024) *Đánh giá an toàn và đặc tính probiotic in-vitro của chủng Bacillus subtilis phân lập từ phân trẻ em*. Tạp chí Y học Việt Nam 537(2).
9. Zhu J, Liu Y, Zhu C, Wei M (2022) *Effects of different drying methods on the physical properties and sensory characteristics of apple chip snacks*. LWT 154: 112829.
10. Shu G, He Y, Chen L et al (2018) *Characterization of freeze-dried Lactobacillus acidophilus in goat milk powder and tablet: optimization of the composite cryoprotectants and evaluation of storage stability at different temperature*. LWT 90: 70-76.
11. Ngo NQA, Dam TX, Nguyen TK et al (2024) *Isolation, Characterization, and Assessment of Probiotic Properties of Bacillus clausii Isolated from Children's Stools in a Northern Province of Vietnam*. Jordan Journal of Pharmaceutical Sciences 17(4): 638–658.
12. Golnari M, Bahrami N, Milanian Z et al (2024) *Isolation and characterization of novel Bacillus strains with superior probiotic potential: comparative analysis and safety evaluation*. Sci Rep 14: 1457.
13. Zeng C, Li J, Shi J et al (2025) *Modulating the Physical Form of Mannitol Crystallizing in Frozen Solutions: The Role of Cosolute and Processing*. Mol Pharmaceutics. doi: 10.1021/acs.molpharmaceut.4c01481.
14. Li J, Wang H, Wang L et al (2024) *Stabilization effects of saccharides in protein formulations: A review of sucrose, trehalose, cyclodextrins and dextrans*. Eur J Pharm Sci 192: 106625.
15. Jia B, Allai L, Li C et al (2024) *A review on the functional roles of trehalose during cryopreservation of small ruminant semen*. Front Vet Sci 11: 1467242.