Low-Temperature Vacuum Spray Drying of Live Biotherapeutic Products

Formulation, Process Development and Data-Driven Analysis Using Probiotic Bacteria

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2026

MASTER THESIS

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Published by
Department of Process and Life Science Engineering
Faculty of Engineering LTH, Lund University
P.O. Box 118, SE-221 00 Lund, Sweden

Publicerad av
Institutionen för processteknik och tillämpad biovetenskap
Lunds Tekniska Högskola, Lunds universitet
Box 118, 221 00 Lund

Subject: KLTM06 Degree Project in Food Engineering, Nutrition and Food Chemistry
Division: Division of Food and Pharma
Supervisor: Anna Fureby
Examiner: Marie Wahlgren

Ämne: KLTM06 Examensarbete i livsmedelsteknik, nutrition och livsmedelskemi
Avdelning: Division of Food and Pharma
Huvudhandledare: Anna Fureby
Examinator: Marie Wahlgren

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by

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Abstract

Low-temperature vacuum spray drying (VSD) is a potential drying approach for probiotic and live biotherapeutic product (LBP) formulations. This thesis investigated VSD for *Limosilactobacillus reuteri* DSM 17938 as a model probiotic strain, with the aim of identifying a feasible lab-scale operating window and using data-driven analysis to support process understanding.

Sucrose–maltodextrin formulations were processed using a Pilotech YC-2000 vacuum spray dryer under varied chamber pressures, inlet temperatures, and formulation compositions. The dried powders were characterised by outlet temperature, water activity, moisture content, powder recovery, particle morphology, particle size distribution, and bacterial viability. Bacterial viability was assessed by CFU counting before drying, after drying, and after 18 days of room-temperature storage. Empirical and machine-learning-assisted models were also applied to explore relationships between process variables, powder properties, and bacterial survival.

Across the bacteria-containing batches, outlet temperature ranged from 38.6 to 65.8°C, water activity from 0.145 to 0.272, and after-drying viability from approximately 3% to 31%. Higher outlet-temperature conditions generally reduced water activity but were associated with greater viability loss, whereas milder drying improved after-drying viability but did not always produce the lowest water activity. Batch 0316A, produced at 60 kPa, 80°C inlet temperature, and a 90:10 sucrose:maltodextrin ratio, showed the most favourable overall performance, with approximately 26% after-drying viability, 81% storage retention, and 21% final survival.

Data-driven analysis supported this operating-window interpretation. Outlet temperature was described by an empirical linear model, water activity was analysed using Ridge regression, and after-drying viability was explored using Random Forest modelling. The modelling results indicated that bacterial survival was strongly linked to the outlet-temperature-related drying state, but remained exploratory due to the limited dataset size.

Overall, this study demonstrates that low-temperature VSD can produce *L. reuteri*-containing powders with measurable viability after drying and short-term storage, and identifies a promising process–formulation region for further optimisation of probiotic and LBP drying processes.

Acknowledgements

First of all, I would like to express my sincere gratitude to my supervisor, Anna Fureby, for her patient guidance, continuous support and trust throughout this thesis project. Her supervision provided me with the opportunity to work on a challenging and interdisciplinary topic involving vacuum spray drying, probiotic formulation and process development. I am especially grateful for her openness and encouragement when I explored the use of data analysis and machine-learning-based approaches to support process understanding. This freedom and trust allowed me to develop the project not only as an experimental study, but also as a broader learning experience connecting formulation science, process engineering and data-driven analysis. Without her support, it would have been much more difficult for me to navigate both the experimental work and the methodological development of this thesis.

I would also like to thank Marie Wahlgren for her support during my master's studies and for serving as the examiner of this thesis. I am also grateful for her encouragement and for kindly acting as a referee for my future academic applications. Her trust and support have been very important to me throughout this period.

I am also grateful to Shuai Bai, Hans Bolinsson and Daniel Espinoza for their helpful support and advice at different stages of this project. Shuai provided useful discussions and practical suggestions related to probiotic formulation, microbiological work and experimental planning. Hans offered technical assistance and helpful advice in the laboratory. Daniel provided valuable feedback on my early data-analysis and machine-learning workflow, and his comments on model evaluation, reproducibility, PCA and decision-focused metrics helped me think more critically about how data-driven methods should be used and reported in a small experimental dataset.

After completing the writing of this thesis, and as I am about to finish my master's studies, I would also like to leave a few words for myself. When I first came to Sweden in 2024, that year's Nobel Prizes seemed to mark a moment when artificial intelligence and data-driven science were being recognised at the highest level. However, what truly brought me closer to programming, data analysis and machine learning was not the prizes themselves, but a difficult but formative personal experience. I have come to see that period as a gift that Sweden gave me, and my later exploration of machine learning as one of the flowers that grew from it.

During this thesis, I gradually realised that machine learning should not be treated as a

fashionable label or a decorative tool. In small experimental datasets, using a complex model only to obtain an impressive number can easily become misleading. At the same time, I also learned that many important engineering and biological problems cannot be fully described by simple first-principles equations alone. The survival of bacteria after drying and storage is one such problem. It is shaped by formulation, drying history, biological stress, storage conditions and experimental variation. In this sense, viability is not only a physical response, but also a statistical outcome under uncertainty.

This understanding changed the way I think about data-driven methods. Their value is not to replace mechanistic understanding, but to help explore complex responses when the mechanism is incomplete, the data are limited, and decisions still need to be made. In *Ars Conjectandi*, Jakob Bernoulli already reflected on the difficulty of reasoning about uncertain real-world events when all possible causes and outcomes cannot be fully enumerated, using examples such as disease and weather. More than three centuries later, fields such as epidemiology and weather forecasting show that complex phenomena once regarded as almost impossible to predict can gradually become objects of statistical description, modelling and computational prediction through accumulated observations and improved methods. Reading Dario Amodei's *Machines of Loving Grace* during this period also made me think more about how artificial intelligence may reshape the way biological and technological progress is made. In this broader sense, the viability and stability of live microorganisms after drying may also be viewed as complex outcomes that require both mechanistic understanding and data-driven learning. Perhaps one day, with larger and higher-quality datasets accumulated across processes, materials, quality attributes and production batches, each manufacturing run can become not only a production event, but also a source of learning for prediction, optimisation and process understanding.

Finally, I would like to thank my family and friends for their patience, support and understanding during this intense period. Their support gave me the stability to complete this work. Looking back, this thesis was not only a study of low-temperature vacuum spray drying, but also a record of how I learned to connect pharmaceutical technology, process engineering, microbiology, machine learning and data analysis into one way of thinking.

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Abbreviations

ANN	Artificial neural network
A_w	Water activity
CFU	Colony-forming unit
CV	Cross-validation
D_{10}	Particle diameter below which 10% of the particle volume is found
D_{50}	Median particle diameter
D_{90}	Particle diameter below which 90% of the particle volume is found
LAB	Lactic acid bacteria
LBP	Live biotherapeutic product
LOOCV	Leave-one-out cross-validation
MAE	Mean absolute error
ML	Machine learning
MLP	Multilayer perceptron
MRS	de Man, Rogosa and Sharpe
QbD	Quality by Design
QPS	Qualified Presumption of Safety
R^2	Coefficient of determination
RMSE	Root mean square error
SEM	Scanning electron microscopy
T_g	Glass transition temperature
T_{in}	Inlet temperature
T_{out}	Outlet temperature
VSD	Vacuum spray drying
w/v	Weight per volume
w/w	Weight per weight

1 Introduction

1.1 Probiotics and Live Biotherapeutic Products

Probiotics are commonly defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Kechagia et al., 2013). They are widely studied for their roles in supporting gut microbial homeostasis and modulating host immune function, with reported benefits that are often strain- and dose-dependent and vary across indications (Hill et al., 2014). In parallel with ongoing scientific research, probiotics have also undergone rapid industrial translation and commercialization, reflected in the expanding market size and growth projections. The global probiotics market was valued at USD 90.56 billion in 2025 and is estimated to grow to USD 97.28 billion in 2026 (Mordor Intelligence, 2026). Building on the long history of traditional probiotics in foods and supplements, a broader range of live microorganisms is now being developed and formulated for human use; when such products are intended to prevent, treat, or cure disease, they fall into the more regulated category of LBPs (Min et al., 2025). The Food and Drug Administration (FDA) defines LBPs as “a biological product that: 1) contains live organisms, such as bacteria; 2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3) is not a vaccine” (Food and Drug Administration (FDA), 2016). Depending on intended use and claims, products containing live microorganisms may be regulated either as food supplements or as drugs; once therapeutic claims are made, LBPs are typically treated as biological medicinal products under more stringent regulatory pathways (Pan et al., 2025). In this context, demonstrating safety and efficacy for a defined indication, while ensuring consistent quality and functional performance of living organisms, becomes a central development challenge (Min et al., 2025).

In this thesis, *Limosilactobacillus reuteri* DSM 17938 (BioGaia AB, Sweden) is used as the model probiotic strain. *L. reuteri* is a well-established member of lactic acid bacteria (LAB) and is widely used in commercial probiotic products (Ramírez et al., 2025). *L. reuteri* strains are noted for their ability to produce the antimicrobial compound reuterin, and this species has been granted Qualified Presumption of Safety (QPS) status at the species level (Boyte et al., 2025). Specifically, DSM 17938 has been the subject of an FDA GRAS notice (GRN No. 254) submitted by BioGaia AB, supporting its use as a food ingredient (U.S. Food & Drug Administration, n.d.).

Using DSM 17938 therefore provides an industrially relevant model to study how formulation matrices and low-temperature drying conditions influence viability and product performance.

1.2 Drying Technologies for Probiotic and LBP Formulations

For probiotic and LBP products, a key formulation and product-development requirement is to achieve a stable and practical dosage form that preserves microbial viability during distribution and storage. Many commercial probiotic preparations are traditionally formulated in liquid forms (G. Wang et al., 2022), which can impose stability constraints and often require refrigerated storage and a refrigerated supply chain (Harrison et al., 2015; Poddar et al., 2014). Consequently, converting liquid cultures into dry solid dosage forms has become a practical requirement for storage, distribution, and downstream handling (G. Wang et al., 2022). In support of this, dehydrated probiotics exhibit enhanced long-term viability and can be incorporated into low-moisture food matrices, which also possess high stability at refrigeration and ambient temperature (Marcial-Coba et al., 2019). Dry preparations can also enable convenient processing steps such as blending, capsule filling, and reconstitution, and storing bioproducts in dry form is often more economical and convenient than frozen storage (Harrison et al., 2015; Poddar et al., 2014). However, drying imposes major stress on microorganisms, with the cytoplasmic membrane being a primary target. Water removal perturbs membrane hydration and lipid packing, which can destabilize the membrane and reduce viability (Bai, 2024).

In pharmaceutical and biologics manufacturing, freeze-drying is a mature option for stabilising labile materials (Osanlóo et al., 2024). The process typically consists of freezing, primary drying, and secondary drying to achieve low residual moisture and improve storage stability (Klepzig et al., 2020; Nowak & Jakubczyk, 2020). In practice, freeze-drying remains widely used for sensitive biologics because it enables drying at low product temperatures and often supports long-term stability of labile formulations. Nevertheless, it is an inherently batch-based operation with long cycle times and high energy demand, and it can become a throughput bottleneck at manufacturing scale (Fiedler et al., 2023; Patel & Pikal, 2011; Sharma et al., 2021). By contrast, conventional spray drying is continuous and scalable, converting a liquid feed into powder via atomization, rapid solvent evaporation in a hot gas stream, particle formation, and product collection (Milanesi et al., 2025). However, for probiotic bacteria, a major disadvantage of spray drying is the loss of viability caused by thermal, osmotic, and oxidative stresses during and after dehydration

(Santivarangkna et al., 2008). From a process perspective, spray drying enables high productivity and well-controlled powder properties, but the elevated drying temperatures commonly applied can be detrimental for heat-sensitive biological systems unless process conditions and formulation protection are carefully optimised (De Melo Ramos et al., 2019). More broadly, in food-grade bacterial applications, spray drying is often faster and less energy-intensive than freeze-drying, yet maintaining high viability remains challenging for sensitive strains (Huang et al., 2017).

These trade-offs motivate interest in an intermediate drying route that retains spray drying's productivity while lowering the thermal burden for heat-sensitive systems. Low-temperature vacuum spray drying is a promising approach in this context. Under reduced chamber pressure, the boiling point of water decreases, so solvent removal can proceed at substantially lower product temperatures than in atmospheric spray drying (De Melo Ramos et al., 2019; Islam et al., 2015). Importantly, VSD retains the atomization-based droplet drying principle characteristic of spray drying, thereby preserving key powder-processing advantages while improving thermal gentleness (Ramos et al., 2021). This positioning between freeze-drying and conventional spray drying is particularly relevant for probiotic and LBP formulations, as it may widen the feasible operating window for viability preservation.

1.3 Vacuum Spray Drying Process Development and Operating Window Definition

In this thesis, VSD is approached as a process development task aimed at establishing a reproducible and manufacturable low-temperature drying route for probiotic and LBP formulations. Rather than optimizing a single endpoint, the objective is to define an operating window in which biological performance and practical manufacturability can be balanced, consistent with Quality by Design (QbD) principles for establishing robust process boundaries (Sharma et al., 2021). Because spray-drying-based processes are inherently sensitive to multiple interacting variables, operating-window definition in the present system requires consideration of both process settings and formulation composition, together with the key responses used to evaluate drying outcome (Muñoz López et al., 2024; Wahab et al., 2025).

Accordingly, the operating window in this thesis is defined by linking key controllable inputs—including chamber pressure, inlet temperature, and formulation composition—to the responses most relevant to the bacteria-containing VSD system.

These include viability measured by colony-forming unit (CFU) counting and water activity (Aw) as the most critical responses, together with moisture content, outlet temperature (Tout), powder recovery, and powder properties relevant to handling and downstream use.

1.3.1 Operating window constraints in vacuum spray drying

For probiotic and LBP formulations, defining an operating window in VSD starts from a practical constraint: probiotic bacteria are often heat-sensitive during spray-drying-type processing (Liu et al., 2015), so higher product temperature exposure can reduce viability, yet overly mild drying can leave powders with elevated moisture and water activity, which undermines handling and storage stability (Umaña et al., 2025). In spray drying of LAB, powder temperature and water activity are commonly treated as key post-drying parameters (Martins et al., 2019), and selecting suitable drying conditions often requires balancing temperature-related stress against moisture-related stability requirements.

Inlet temperature (T_{in}) is one of the most influential process parameters in spray-drying-type operations because drying is driven by contacting atomized droplets with a hot gas stream, and the thermal environment governs evaporation and the short thermal history experienced by the solids (Wahab et al., 2025). Building on this engineering basis, pharmaceutical spray-drying studies further link T_{in} to evaporation rate and drying kinetics, which can promote rapid surface skin formation and thereby affect particle morphology. Faster drying associated with higher T_{in} has also been reported to increase the glass transition temperature (T_g) of the resulting solids, supporting a less sticky and more physically stable powder state, whereas lower T_{in} tends to yield lower-T_g, stickier particles that constrain the operating window (Adhikari et al., 2005; Wahab et al., 2025). At the same time, although spray drying involves exposure to a hot gas stream, it is still widely applied to heat-sensitive products because drying occurs on a short timescale and, during active evaporation, the droplet or wet-particle temperature may remain closer to the wet-bulb temperature than to the inlet gas temperature (Harrison et al., 2015; Nik Abd Rahman et al., 2024).

VSD addresses temperature sensitivity primarily through reduced chamber pressure. Lower pressure lowers the evaporation temperature of water, enabling solvent removal at lower product temperatures than in atmospheric spray drying (De Melo Ramos et al., 2019; Islam et al., 2015). However, reduced pressure does not simply lower temperature; it also alters the heat and moisture-transfer conditions in the drying chamber, which may reduce the evaporation rate and overall drying rate unless

inlet temperature and related process conditions are adjusted accordingly (Yang, 2025). Consistent with this trade-off, vacuum spray drying studies on carbohydrate systems have reported higher moisture contents in VSD powders under certain conditions compared with conventional spray drying, indicating a risk of producing powders with higher residual moisture when drying intensity is insufficient (De Melo Ramos et al., 2019).

Accordingly, this thesis treats chamber pressure and inlet temperature as the primary operating-window parameters, because their combination governs the balance between thermal gentleness and drying intensity. Within this framework, CFU-based viability is treated as the primary biological response, as the preservation of live microorganisms is central to the function of probiotic and live biotherapeutic formulations (A. Wang & Zhong, 2024). Outlet temperature is monitored as a practical indicator of the overall drying regime and maximum temperature exposure during processing. Water activity and moisture content are used to indicate whether drying is sufficient for stable powder handling and storage (Martins et al., 2019).

1.3.2 Formulation considerations relevant to operating window definition

In probiotic process development, formulation is not merely a supporting variable but an integral part of operating window definition (Vehring, 2008; A. Wang & Zhong, 2024). While chamber pressure and inlet temperature define the thermal and drying regime of vacuum spray drying, formulation influences how microorganisms experience that regime at the droplet and matrix level. Protective excipients therefore influence not only the immediate stress imposed during drying (Broeckx et al., 2017), but also the physical state and practical handling properties of the resulting powder (A. Wang & Zhong, 2024).

In this thesis, sucrose–maltodextrin blends were selected as the main formulation axis because they provide a practical balance between biological protection and powder processability (Bai, 2024; Yang, 2025). Disaccharides such as sucrose are widely used in drying formulations due to their stabilising effects on cell membranes and other sensitive biological structures during dehydration (Lim et al., 2026; A. Wang & Zhong, 2024). In contrast, maltodextrin contributes mainly to matrix formation and powder stability by supporting a less sticky solid state and improving handling properties after drying (Zhang et al., 2018). Earlier placebo studies performed on the same VSD platform showed that formulation variables, including solids concentration and sucrose–maltodextrin composition, significantly affected moisture-related properties and powder morphology under defined drying conditions (Yang, 2025). These

findings informed the formulation strategy used in the present study, including the selection of sucrose–maltodextrin blends as the main formulation axis.

1.4 Data-Driven Modelling for Drying Process Development

Alongside experimental development, there is growing interest in data-driven and hybrid modelling approaches for complex drying processes, particularly in spray drying and related pharmaceutical drying operations (Klepzig et al., 2020; Muñoz López et al., 2024; Wahab et al., 2025). Spray drying involves coupled heat and mass transfer, droplet evaporation, and particle formation, and several mechanistic and semi-empirical models have been proposed to describe these physical phenomena (Chegini et al., 2008; Hu et al., 2026; Muñoz López et al., 2024; Wahab et al., 2025). However, recent reviews in pharmaceutical spray drying also emphasize that purely mechanistic modelling remains challenging because of the computational cost, the difficulty of experimental validation, and the limited applicability of such models under complex operating and formulation conditions (Wahab et al., 2025). For this reason, data-driven methods, particularly machine learning (ML), have received increasing attention as complementary tools for process optimisation, process monitoring, and predictive support (Muñoz López et al., 2024; Wahab et al., 2025).

From a QbD perspective, process development aims to establish relationships among material attributes, process parameters, and critical quality attributes (CQAs), thereby enabling the definition of a robust design space and operating window (International Council for Harmonisation (ICH), 2009). Under such a framework, the role of data-driven modelling is not to replace mechanistic understanding, but to support the identification of these relationships when experimental exploration is constrained by high cost and the multidimensional nature of the process space. This challenge is particularly relevant in drying processes for biologics, where material availability is limited and systematic experimentation becomes impractical. In this context, data-driven approaches have been explored to facilitate design-space development and process understanding in spray drying systems, including applications in protein drying and process monitoring (Fiedler et al., 2023; Muñoz López et al., 2024).

Within the present thesis, different process responses are treated using different levels of modelling complexity. Some outputs in drying, such as outlet temperature, are largely governed by heat and mass transfer and can often be described using empirical correlations or relatively simple regression models (Chegini et al., 2008; Muñoz López et al., 2024). In contrast, bacterial viability is more difficult to represent using a

single mechanistic relationship. Previous studies on spray drying of lactic acid bacteria suggest that survival is not determined solely by process-air temperatures, but is more closely associated with powder-state conditions such as water activity (Martins et al., 2019). Accordingly, viability is treated here as a complex response resulting from the combined effects of process conditions, formulation-dependent protection, and multiple biological damage mechanisms. This motivates the use of data-driven modelling as a practical tool for exploring input–output relationships in the bacteria-containing VSD system.

At the same time, the limitations of machine learning must be explicitly acknowledged. Reviews of pharmaceutical spray drying emphasize that ML models depend strongly on data quality and may not generalize well beyond the training domain, particularly when formulation and process conditions vary (Wahab et al., 2025). This consideration is especially relevant in the present study, where the bacteria-containing VSD system is experimentally demanding and the available dataset is inherently limited in size. Under such conditions, ML models may capture patterns within the observed data without guaranteeing robust extrapolation to new operating conditions. Accordingly, ML is used here primarily as an exploratory tool to support analysis rather than as a standalone predictive framework.

1.5 Study Aim and Scope

The overall aim of this thesis is to develop and evaluate a low-temperature VSD process for probiotic and LBP formulations, using *Limosilactobacillus reuteri* DSM 17938 as a model strain. The study focuses on defining a practical lab-scale operating window in which bacterial viability can be maintained while achieving sufficiently low water activity, acceptable moisture content, and suitable powder properties for handling and storage.

To this end, the effects of key process variables, especially chamber pressure and inlet temperature, are investigated together with formulation variables such as excipient composition and solids content. CFU-based viability and water activity are treated as the primary responses, while moisture content, outlet temperature, powder recovery, and powder properties are used as supporting indicators of drying performance and manufacturability.

In addition, the thesis aims to establish a structured data basis for process understanding in the bacteria-containing VSD system. Experimental data generated during screening and process development are used not only to identify feasible

operating regions, but also to support exploratory data-driven analysis of formulation–process–response relationships. In this context, data-driven methods are applied as supportive tools for interpretation, comparison, and process-window screening, rather than as standalone predictive endpoints.

Overall, the thesis seeks to provide both an experimentally grounded operating-window framework and an initial data-driven basis for future development of probiotic and LBP drying processes.

2 Materials and Methods

2.1 Materials

The materials used in this study are listed in **Table 1**.

Table 1. *Materials used for the production and characterisation of powders*

Material	Supplier
Limosilactobacillus reuteri DSM 17938	BioGaia
Sucrose	Sigma-Aldrich
Maltodextrin Glucidex® 12	Roquette
Sodium chloride	VWR Chemicals
MRS agar	Merck Millipore
Milli-Q water	Milli-Q IQ 7000 system, Merck Millipore
Lamp oil	Petromax Alkan
Sorbitan monolaurate (Span® 20)	Sigma-Aldrich

2.2 Experimental Design and Study Overview

The experimental work consisted of five preliminary placebo runs followed by bacteria-containing vacuum spray drying batches. The placebo runs were performed using sucrose–maltodextrin formulations without bacteria to confirm the practical workflow for feed preparation, VSD operation, powder collection, and basic powder characterisation. These runs also provided reference data for the drying behaviour of the excipient matrix before introducing live bacteria.

The main experimental dataset was generated using formulations containing *L. reuteri*. The tested process and formulation variables included chamber pressure, inlet temperature, total solids concentration, and sucrose-to-maltodextrin ratio. The initial 14 bacteria-containing runs were arranged as a structured screening set to explore the effects of pressure, inlet temperature, and formulation composition. Four additional follow-up batches were then performed around selected intermediate conditions to further examine the operating region associated with acceptable drying performance and bacterial survival.

For each batch, the experimental process was recorded, including the main process settings, formulation composition, and sample handling steps. The corresponding measured responses included outlet temperature, water activity, moisture content, powder recovery, after-drying bacterial viability, and 18-day storage stability. The

experimental batches and main experimental variables are summarised in **Table 2**.

Table 2. *Overview of experimental batches and main experimental variables.*

Batch ID	Pressure (kPa)	Solids (%, w/w)	Sucrose in solids (%)	Maltodextrin in solids (%)	T_{in} (°C)
0203 test	81.3	42.25	70	30	100
0204 test	81.3	30	80	20	100
0205 test	81.3	30	90	10	100
0206 test	81.3	30	70	30	100
0209 test	81.3	42.25	70	30	70
0309A	80	30	70	30	70
0309B	80	30	70	30	110
0310A	80	30	90	10	70
0310B	80	30	90	10	110
0311A	60	30	70	30	80
0312A	60	30	70	30	110
0316A	60	30	90	10	80
0316B	60	30	90	10	110
0317A	70	30	80	20	95
0317B	70	30	80	20	95
0323A	80	30	70	30	80
0323B	60	30	70	30	130
0325A	80	30	90	10	80
0325B	60	30	90	10	130
0330A	80	30	90	10	75
0330B	80	30	90	10	85
0331A	80	30	90	10	80
0331B	80	30	70	30	75

2.3 Preparation of Probiotic Feed Suspension

The probiotic feed suspension was prepared by first recovering the bacterial material from the commercial freeze-dried powder and then incorporating the cell-enriched fraction into a sucrose–maltodextrin excipient matrix according to the experimental design. After thorough mixing, the final feed suspension was transferred to the vacuum spray dryer and processed as soon as practical to minimize the time between bacterial exposure to the excipient solution and the start of vacuum spray drying.

2.3.1 Preparation of excipient solution

Sucrose and maltodextrin were weighed according to the target sucrose-to-maltodextrin ratio in the total excipient solids. The tested ratios included 70:30, 80:20 and 90:10 sucrose:maltodextrin. For each standard batch, the excipient solids mass was 15 g and the final feed mass was adjusted to 50 g, corresponding to a target total solids content of 30% (w/w). The excipients were dissolved in Milli-Q water under stirring at room temperature until a homogeneous solution was obtained.

2.3.2 Preparation of bacterial suspension

For each bacteria-containing batch, the freeze-dried *L. reuteri* powder was dispersed in sterile 0.85% NaCl solution in a 50 mL centrifuge tube and mixed until no visible agglomerates remained. The suspension was then centrifuged to obtain a cell-enriched pellet and to remove dissolved matrix components from the original powder. After centrifugation, the supernatant was discarded and the pellet was retained for formulation preparation. The pellet was added to the prepared sucrose–maltodextrin excipient solution and mixed thoroughly to obtain the final feed suspension.

2.3.3 Feed holding before drying

After incorporation of the bacterial pellet, the final feed suspension was mixed thoroughly and used for vacuum spray drying as soon as practical. The time between addition of the sucrose–maltodextrin excipient solution and the start of drying was recorded as the feed holding time. This time was kept below 30 min whenever possible, because prolonged exposure of the bacteria to the sugar-containing excipient solution could allow bacterial metabolism of the sugars.

2.4 Vacuum Spray Drying Process

Vacuum spray drying was performed using a Pilotech YC-2000 vacuum spray dryer. Before each run, the system was assembled and operated according to the established laboratory procedure. The spray pressure was set to 0.15 MPa, the nozzle diameter was 0.7 mm, and the nozzle auto-cleaning interval was set to 20 s. The feed rate was kept constant at 8.8 mL/min for all drying runs.

Before introducing the sample feed, the system was first allowed to reach thermal equilibrium at the target inlet temperature and chamber pressure. Milli-Q water was then pumped through the system until the outlet temperature and operating conditions became stable. After this water-stabilisation step, the feed tube was transferred from water to the prepared probiotic feed suspension. During drying, the inlet temperature

and chamber pressure were regulated by the equipment according to the selected settings, while the outlet temperature was recorded as the main process response. At the end of each run, the feed tube was switched back to Milli-Q water and flushed for approximately 2.5 min to rinse the feed line and nozzle. Operational observations, including total drying time, chamber temperature, wall deposition and powder recovery, were recorded for each batch.

For runs with inlet temperatures of 95°C or higher, the product collection vessel was placed in a room-temperature water bath at system start-up and kept in the water bath throughout the subsequent stabilisation and drying steps. This was used as a practical temperature-control measure to keep the stainless-steel collection vessel near room temperature, approximately 25°C, and to reduce secondary thermal exposure of the dried powder during collection. Ice-water cooling was tested during preliminary trials but was not used in the final protocol because visible condensation was observed, whereas room-temperature water cooling did not lead to visible condensation in the product vessel.

The recovered powder was collected from the product vessel as soon as possible after the end of drying, labelled with the corresponding batch ID and divided into portions according to the subsequent analyses. One portion was used for same-day water activity and moisture content measurements, while other portions were stored for later CFU analysis, 18-day storage testing, and particle characterisation. Samples intended for viability analysis were stored at -80°C, and storage samples were kept sealed and protected from light until further analysis.

2.5 Characterisation of Dried Powders and Biological Performance

The dried powder samples were characterised using both physicochemical and biological measurements. The physicochemical characterisation included outlet temperature, water activity, moisture content, powder recovery, particle morphology, and particle size distribution. Biological performance was evaluated by CFU counting at three stages: in the final feed suspension before drying, in the reconstituted powder after drying, and after 18 days of storage. These measurements were used to assess drying survival, short-term storage stability, and final survival relative to the pre-drying baseline. Selected samples were further analysed by scanning electron microscopy (SEM) to provide supplementary information on particle morphology and formulation-dependent structural differences.

2.5.1 Outlet temperature recording

During each VSD run, the outlet temperature was recorded from the instrument display and the experimental run sheet during the powder-drying stage. After the outlet temperature reached a relatively stable operating range, this range was recorded, and the central value was used as the representative outlet temperature for the corresponding batch.

2.5.2 Water activity measurement

The water activity of the dried powder samples was measured at 20℃ using an Aqualab Decagon 3TE water activity meter (Decagon Devices Inc., Pullman, WA, USA). Before each measurement session, the instrument was checked and calibrated according to the manufacturer's instructions. Approximately 0.5 g of powder was gently placed into the sample cup to cover the bottom surface while avoiding compression or overfilling. Measurements were performed in duplicate, and the mean value was reported for each batch.

2.5.3 Moisture content measurement

Approximately 0.5 g of powder was weighed into an aluminium weighing dish and dried overnight in an oven at 105℃. Each sample was prepared in triplicate. After drying, the samples were cooled in a desiccator for at least 30 min before the final weight was recorded. Moisture content was calculated from the weight loss after drying and expressed as a percentage of the initial powder mass.

2.5.4 Viability measurement

Bacterial viability was quantified by CFU counting before and after vacuum spray drying. For each VSD run, a pre-drying baseline sample was taken from the final feed suspension after thorough mixing and before introduction into the VSD system. The aliquot was serially diluted using sterile 0.85% NaCl solution and plated on MRS agar. Plates were incubated at 37℃ for 48 h before colony counting. Countable plates, generally containing 30–300 colonies, were used for CFU calculation, and the mean value from replicate plates was reported.

For post-drying viability measurement, a defined mass of dried powder was weighed and reconstituted in sterile 0.85% NaCl solution. The reconstituted sample was mixed thoroughly to disperse the powder and was then serially diluted. Appropriate dilution levels were plated on MRS agar in duplicate or triplicate, depending on the available sample amount and plate number. After incubation under the same conditions, colonies were counted from the countable plates, and viable counts were reported for

the dried powder samples.

2.5.5 Short-term storage stability study

Short-term storage stability was evaluated for the bacteria-containing dried powder samples after 18 days of storage. The storage samples were kept sealed at room temperature and protected from light until analysis. After storage, a defined mass of powder was reconstituted in sterile 0.85% NaCl solution, mixed thoroughly, and analysed using the same serial dilution and MRS agar plating procedure described above. Appropriate dilution levels were plated in duplicate or triplicate depending on the available sample amount and plate number. The viable counts after storage were used to calculate storage retention relative to the after-drying CFU value and final survival relative to the pre-drying baseline during data processing.

2.5.6 Particle morphology and particle size distribution

Particle morphology was examined using an Olympus System Microscope (model BX50) equipped with a 10× ocular lens. A small amount of dried powder was dispersed in lamp oil containing 5% (w/v) Span 20 and mixed to obtain a suspension. The suspension was placed in an ultrasonic water bath to reduce visible agglomeration and improve particle dispersion. A small volume of the dispersed suspension was then placed on a glass slide, covered with a cover slip and observed under the optical microscope. Images were acquired using objective lenses at 50× and 100× magnifications, and representative fields of view were photographed for qualitative observation of particle morphology.

Particle size distribution was measured by laser diffraction using a Mastersizer 2000 (Malvern Instruments, UK). The same oil-based dispersion procedure was used for particle size measurement. After confirming that no major visible agglomeration was present under the microscope, the dispersed sample was gradually added dropwise into the dispersant unit of the Mastersizer until the obscuration reached approximately 5–10%. The particle size distribution was calculated using the Mie scattering model. Lamp oil with a refractive index of 1.43 was used as the dispersant, and a particle refractive index of 1.53 was applied for the sucrose–maltodextrin powders. The results were reported as D_{10}, D_{50}, D_{90}, and span.

2.5.7 SEM morphology observation

Selected dried powder samples were analysed by SEM to obtain supplementary qualitative information on particle morphology and formulation-dependent structural differences. Six samples were selected based on sucrose:maltodextrin ratio, drying

conditions and storage behaviour, with the aim of comparing 90:10 and 70:30 formulations under similar or contrasting process conditions. Small amounts of powder were transferred into labelled Eppendorf tubes before SEM preparation. For imaging, the powder was spread onto an area of approximately 1 cm^2 on the SEM sample holder and sputter-coated with a gold conductive layer before observation. The SEM analysis focused on qualitative features including particle shape, surface morphology, aggregation, cracks or collapse-like structures, and possible exposure or embedding of bacterial cells within the dried matrix.

2.6 Data Processing, Statistical Analysis and Modelling

All experimental data were processed using Python to generate cleaned analysis datasets. Raw experimental records were standardised by harmonising variable names, checking units and recalculating derived variables where necessary. For the 2026 bacteria-containing experiments, CFU-derived survival metrics, including after-drying bacterial viability, 18-day storage retention and final survival relative to before drying, were recalculated from the corresponding CFU values to verify internal consistency. Percentage-format variables were generated for reporting and visualisation.

Outlet temperature was modelled as an empirical linear process-state descriptor using the 2026 bacteria-containing dataset. Several linear candidate models with selected interaction terms were compared using full-fit metrics, leave-one-out cross-validation (LOOCV) and 4-fold cross-validation. The selected outlet temperature model was then used to generate the predicted outlet temperature descriptor for downstream water activity and bacterial viability modelling.

For water activity modelling, data from the 2025 and 2026 VSD experiments were combined, and only samples with measured water activity were retained. Given that the datasets were generated using the same VSD platform, with comparable formulation principles and operating procedures, water activity was analysed as a shared physicochemical powder attribute across placebo and bacteria-containing samples. Ridge regression was treated as a regularised linear regression method that can reduce overfitting in limited datasets while retaining coefficient-based interpretation. Model performance was evaluated using 5-fold cross-validation.

For bacterial viability modelling, only the 2026 bacteria-containing dataset was used. An initial model screening was performed across survival-related response variables using multiple feature sets and model types, including mean prediction, linear regression, Ridge regression, Random Forest, and multilayer perceptron (MLP).

Random Forest was treated as a non-linear ensemble tree-based method suitable for exploring non-linear relationships and variable interactions, while MLP was included as a neural-network-based comparator during model screening. After-drying bacterial viability was selected as the main exploratory target because it showed the clearest cross-validated signal among the survival-related responses. Random Forest and MLP configurations were then evaluated using grid search, and the final Random Forest model was selected for interpretation.

Model performance was evaluated using 4-fold cross-validation as the primary evaluation and LOOCV as a secondary check, with coefficient of determination (R^2), root mean square error (RMSE), and mean absolute error (MAE) used as performance metrics. Feature importance was evaluated for the final Random Forest model. A 20% after-drying viability level was used as a visual reference threshold in selected plots to distinguish higher-viability batches. As only 18 bacteria-containing observations were available, the Random Forest interpretation was treated as exploratory.

To support reproducibility, the cleaned analysis datasets, figure outputs, and Python scripts used for data processing, modelling, and visualisation were organised separately and are available from the author upon reasonable request.

3 Results

3.1 Overview of VSD experimental runs and measured responses

The bacteria-containing VSD experiments produced dried powders under different chamber pressures, inlet temperatures and sucrose–maltodextrin formulation compositions. Across the experimental batches, the measured responses included outlet temperature, water activity, moisture content, powder recovery, after-drying bacterial viability and 18-day storage stability. These responses were used to evaluate both the drying performance of the VSD process and the biological performance of the dried probiotic powders.

A condensed overview of the key measured responses is provided in **Table 3**. The outlet temperature ranged from 38.6 to 65.8℃, while water activity ranged from 0.145 to 0.272 and moisture content ranged from approximately 3.6% to 6.3%. After-drying viability varied substantially among batches, from approximately 2.8% to 31%. The 18-day storage retention also differed across samples, ranging from approximately 13% to 93%, while final survival after storage ranged from approximately 0.7% to 21%.

Table 3. *Condensed overview of bacteria-containing VSD batches and key responses.*

Batch ID	Tout (℃)	Aw	Moisture content (%)	After-drying viability (%)	18-day storage retention (%)	Final survival (%)	Powder recovery (%)
0309A	42.7	0.211	4.77	11.6	20.0	2.3	50
0309B	64.9	0.145	3.59	4.5	65.5	2.9	40
0310A	42.8	0.200	4.95	28.8	26.3	7.6	30
0310B	65.8	0.150	4.03	3.6	80.5	2.9	33
0311A	38.9	0.272	6.26	31.0	26.8	8.3	30
0312A	53.0	0.222	5.14	5.4	13.2	0.7	43
0316A	38.6	0.229	5.54	26.5	80.6	21.3	23
0316B	52.8	0.210	5.02	2.8	83.7	2.3	20
0317A	51.6	0.174	4.36	4.7	93.4	4.4	47
0317B	51.8	0.167	4.22	5.3	50.7	2.7	43
0323A	48.3	0.183	4.65	9.1	46.5	4.2	53
0323B	62.6	0.156	4.45	3.2	70.9	2.3	37
0325A	46.8	0.207	5.00	9.2	66.8	6.1	43

Batch ID	Tout (°C)	Aw	Moisture content (%)	After-drying viability (%)	18-day storage retention (%)	Final survival (%)	Powder recovery (%)
0325B	60.5	0.189	4.95	2.9	40.3	1.2	30
0330A	43.5	0.220	5.55	11.8	64.5	7.6	40
0330B	49.4	0.174	4.58	4.7	58.9	2.8	40
0331A	47.2	0.194	3.86	5.5	41.6	2.3	57
0331B	43.9	0.226	5.44	7.0	29.7	2.1	43

The full dataset, including process and formulation variables, powder properties and CFU-derived survival metrics, is provided in **Appendix A**. Particle size distribution results are presented separately in Section 3.4.

3.2 Process temperature response under different VSD conditions

Outlet temperature was recorded as the main process temperature response during vacuum spray drying. Across the bacteria-containing batches, the measured outlet temperature ranged from 38.6 to 65.8°C, while the applied inlet temperature ranged from 70 to 130°C. As shown in **Figure 1**, outlet temperature generally increased with increasing inlet temperature under the tested VSD conditions.

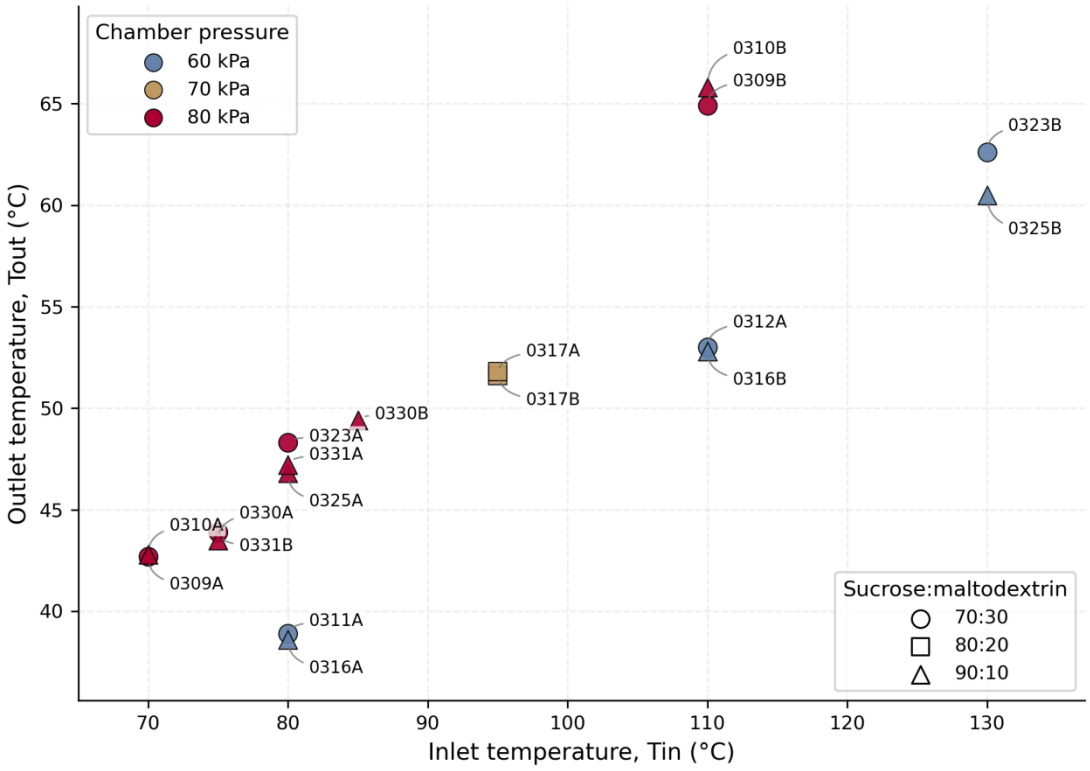

Figure 1. *Outlet temperature response as a function of inlet temperature under different chamber pressure and formulation conditions. Point colour represents*

chamber pressure, while marker shape represents the sucrose:maltodextrin ratio.

The data also indicated a pressure-dependent shift in the outlet-temperature response. The 60 kPa and 80 kPa conditions appeared to follow similar increasing trends with inlet temperature, but at different outlet-temperature levels. At comparable inlet temperatures, batches operated at lower chamber pressure generally showed lower outlet temperatures, while the 70 kPa batches were positioned between the 60 and 80 kPa groups. This pressure effect was particularly clear around an inlet temperature of approximately 80°C. Under this condition, batches operated at 60 kPa showed outlet temperatures of 38.6–38.9°C, whereas batches operated at 80 kPa gave outlet temperatures of 46.8–48.3°C. These results indicate that chamber pressure had a clear influence on the process-temperature state, in addition to the effect of inlet temperature.

The effect of formulation composition on outlet temperature appeared smaller than the effects of inlet temperature and chamber pressure. At matched or similar process settings, the 70:30 and 90:10 sucrose:maltodextrin formulations generally produced comparable outlet temperatures, although small formulation-dependent differences were observed in some paired conditions.

3.3 Water activity and moisture content

Water activity and moisture content were measured as key moisture-related powder properties after vacuum spray drying. Across the bacteria-containing batches, water activity ranged from 0.145 to 0.272, while moisture content ranged from 3.59% to 6.26% (**Table 3**). Most samples showed water activity values between approximately 0.17 and 0.23 and moisture contents between approximately 4% and 5.5%.

The relationship between water activity and moisture content is shown in **Figure 2**. Overall, samples with higher moisture content tended to show higher water activity. For example, the highest water activity was observed for batch 0311A, which also showed the highest moisture content. In contrast, batches such as 0309B and 0310B showed comparatively low water activity together with lower moisture contents.

These results show that water activity and moisture content provided complementary descriptions of the moisture state of the dried powders rather than interchangeable measurements. This was supported by the paired batches 0317A and 0317B, which were produced under the same nominal process and formulation conditions and showed only minor differences in both water activity and moisture content. However,

batches 0325A and 0331A, which were also produced under the same nominal conditions, showed similar outlet temperatures and water activity values but a clearer difference in measured moisture content. Their outlet temperatures were 46.8 and 47.2 °C, and their water activity values were 0.207 and 0.194, respectively, whereas their moisture contents were 5.00% and 3.86%. This difference may reflect the distinct information provided by the two measurements, but experimental uncertainty in the moisture-content method or non-uniform water distribution within the powder may also have contributed. Therefore, water activity was used as the primary moisture-related response in later analysis of powder stability and bacterial survival, while moisture content was retained as a complementary powder property.

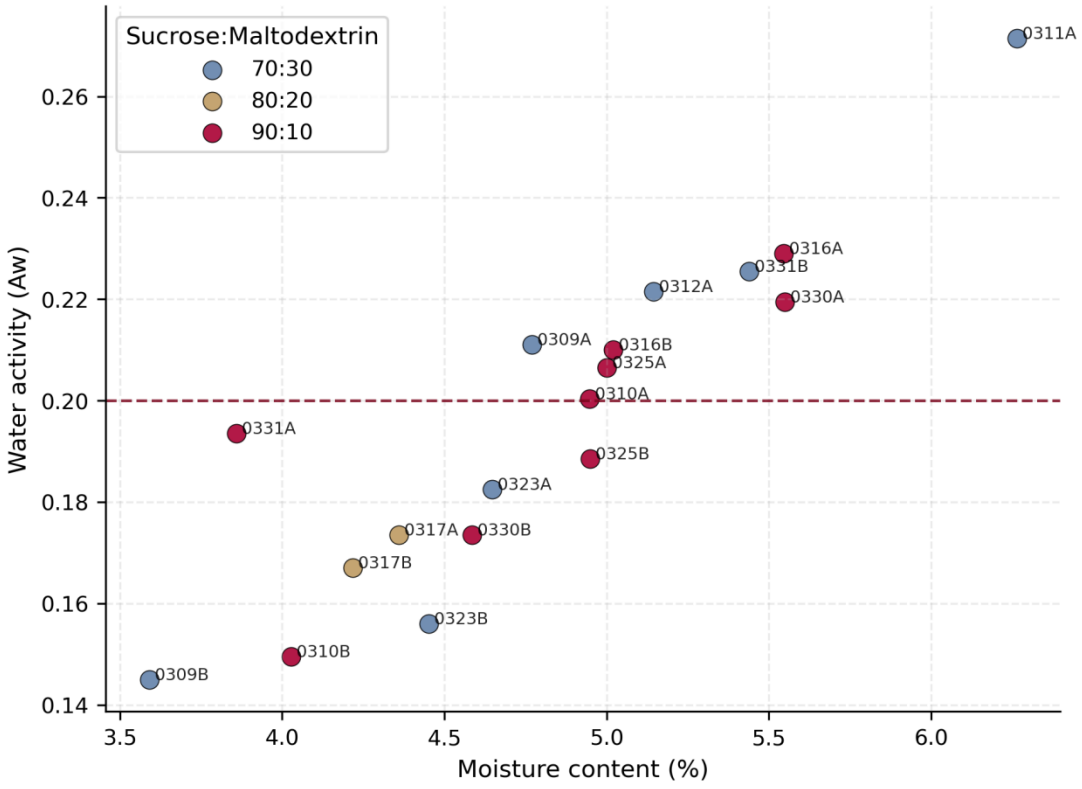

Figure 2. *Relationship between water activity and moisture content of VSD powders. The dashed line indicates Aw = 0.20 as a reference level for low-Aw powders.*

3.4 Particle morphology and particle size distribution

Particle morphology and particle size distribution were examined to assess whether the dried VSD powders showed visible structural differences under the tested formulation and process conditions. Optical microscopy was used as an initial qualitative assessment of particle appearance and aggregation, while laser diffraction was used to quantify particle size distribution. These measurements were used to

support the interpretation of powder quality and to evaluate whether morphology or particle size differed noticeably among the produced powders. Selected samples were further considered for SEM observation to provide higher-resolution information on particle surface morphology and matrix structure.

3.4.1 Optical microscopy observation

Representative optical microscopy images of selected dried powders are shown in **Figure 3**. Overall, the powders appeared as dispersed carbohydrate-based particles with some degree of particle overlap and aggregation. At the resolution of optical microscopy, the selected samples showed broadly similar particle appearance, and no clear formulation-dependent morphological difference could be identified. Occasional larger clusters were observed in some images, suggesting that aggregation or incomplete dispersion may have contributed to broader particle size distributions in selected samples.

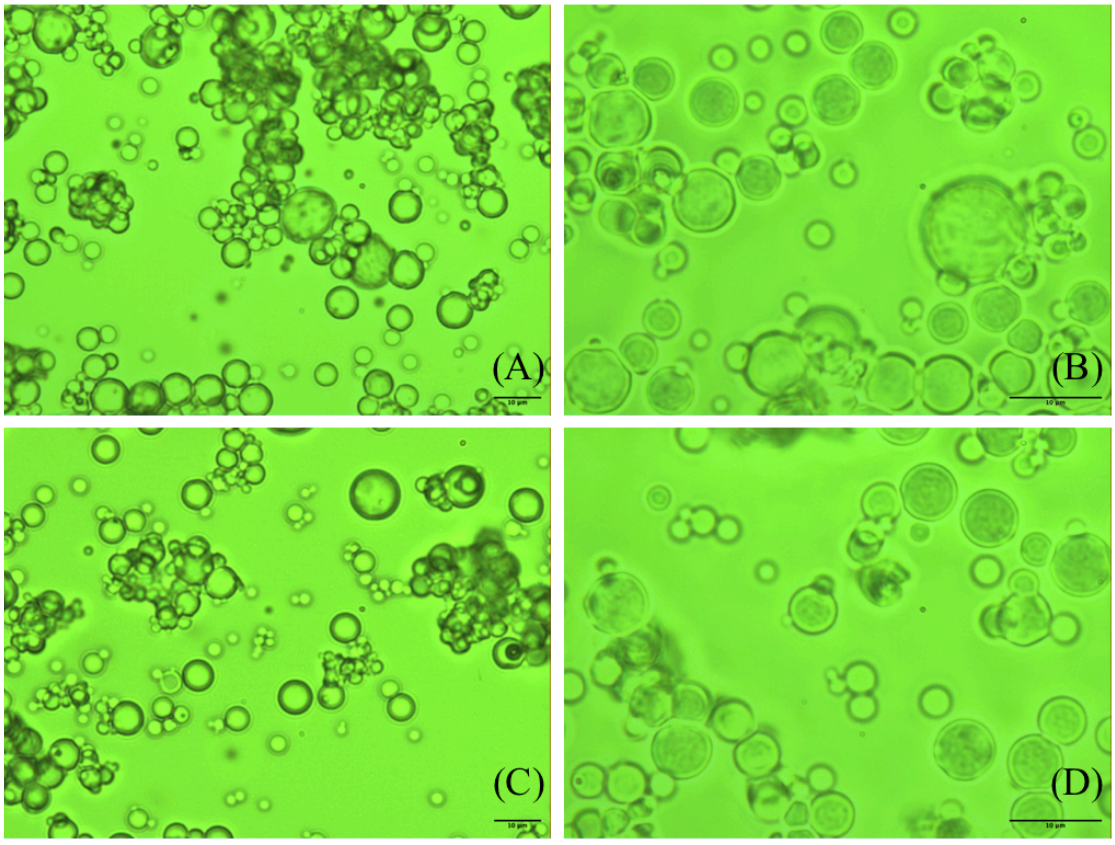

Figure 3. *Representative optical microscopy images of selected dried VSD powders. (A) Batch 0323B observed at 50× magnification; (B) batch 0323B observed at 100× magnification; (C) batch 0325A observed at 50× magnification; (D) batch 0325A observed at 100× magnification. Samples were dispersed in lamp oil medium before observation. Scale bars indicate 10 µm.*

3.4.2 Particle size distribution

Particle size distribution results are summarised in **Table 4**. Most samples showed D_{10} values of approximately 4–6 μm and D_{50} values of approximately 10–16 μm, indicating that the central particle size range was relatively similar across the tested batches. D_{90} values were generally below 55 μm, with the exception of batch 0309A, which showed a much higher D_{90} of 234.021 μm and a span of 11.936. This broader distribution was likely caused by a small fraction of agglomerates, as the median particle size of 0309A remained within the range of the other batches. Apart from this outlying distribution, particle size did not appear to be a major differentiating feature among the VSD powders.

Table 4. *Particle size distribution of dried VSD powders.*

Batch ID	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)	Span
0309A	6.667	19.048	234.021	11.936
0309B	4.755	13.557	29.604	1.833
0310A	4.901	15.903	55.127	3.158
0310B	4.138	13.209	43.655	2.992
0311A	4.649	12.668	31.085	2.087
0312A	4.234	11.486	27.61	2.035
0316A	5.548	18.061	42.823	2.064
0316B	5.01	12.971	28.414	1.804
0317A	4.829	14.038	31.664	1.912
0317B	4.072	11.62	31.193	2.334
0323A	5.531	14.186	34.653	2.053
0323B	4.886	11.052	23.419	1.677
0325A	6.154	15.396	32.382	1.704
0325B	4.136	11.939	29.195	2.099
0330A	4.934	11.504	25.653	1.801
0330B	4.316	10.826	24.276	1.844
0331A	4.794	11.57	26.15	1.846
0331B	3.994	8.454	19.266	1.806

3.4.3 SEM morphology observation

SEM imaging was performed to further examine the surface morphology of selected dried VSD powders. During SEM preparation and observation, some samples showed clear particle softening, fusion or extensive aggregation, which was likely influenced by moisture uptake during sample handling. Therefore, SEM images were interpreted

qualitatively, and representative regions with well-preserved particles were selected for presentation. Representative images from batch 0323B and batch 0325A are shown in **Figure 4**. These samples were selected because they provided relatively clear and well-preserved particle images during SEM observation.

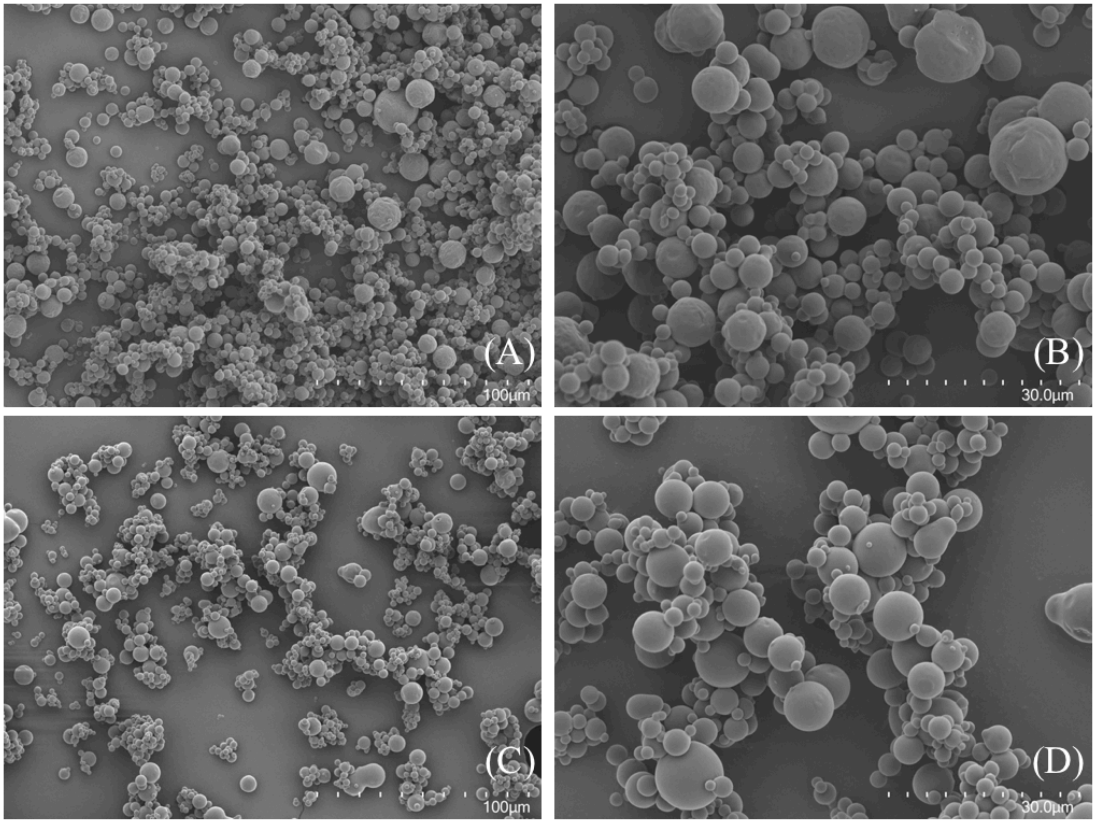

Figure 4. *Representative SEM images of selected dried VSD powders. (A) Batch 0323B at lower magnification; (B) batch 0323B at higher magnification; (C) batch 0325A at lower magnification; (D) batch 0325A at higher magnification. The images show the overall particle morphology and surface appearance of vacuum spray-dried probiotic powders. Scale bars are shown in each panel.*

In the selected images, both samples showed mainly rounded or spherical particles with variable particle sizes. Individual particles could be clearly distinguished, and no obvious collapsed or highly irregular particle morphology was observed in the representative regions. These observations were broadly consistent with the optical microscopy results, while SEM provided clearer information on particle surface appearance and individual particle structure.

Batch 0323B and batch 0325A represented different sucrose:maltodextrin formulations, corresponding to 70:30 and 90:10, respectively. Based on the available SEM images, no clear formulation-dependent difference in overall particle shape or surface appearance was observed. Therefore, the better biological performance observed in some 90:10 samples could not be directly explained by a visibly different

particle morphology based on SEM alone.

3.5 Bacterial survival during drying and short-term storage

Bacterial survival was evaluated at three stages: before drying, after vacuum spray drying and after 18 days of storage. The before-drying CFU value was used as the baseline for calculating after-drying viability, while 18-day storage retention was calculated relative to the after-drying CFU value. Final survival was calculated relative to the before-drying baseline after 18 days of storage. These three metrics were used to distinguish survival loss during drying from additional loss during short-term storage.

The survival trajectory across drying and storage is shown in **Figure 5**. All batches showed a decrease in relative survival after vacuum spray drying, but the magnitude of this loss differed substantially among batches. After-drying viability ranged from 2.8% to 31% (**Table 3**). The highest after-drying viability was observed for 0311A, 0310A, and 0316A, whereas batches such as 0316B, 0325B, 0323B, and 0310B showed the lowest after-drying viability. Further decreases were observed after 18 days of storage, although the extent of this additional loss varied among batches. Final survival after 18 days ranged from 0.7% to 21% (**Table 3**), with 0316A showing the highest final survival among all batches.

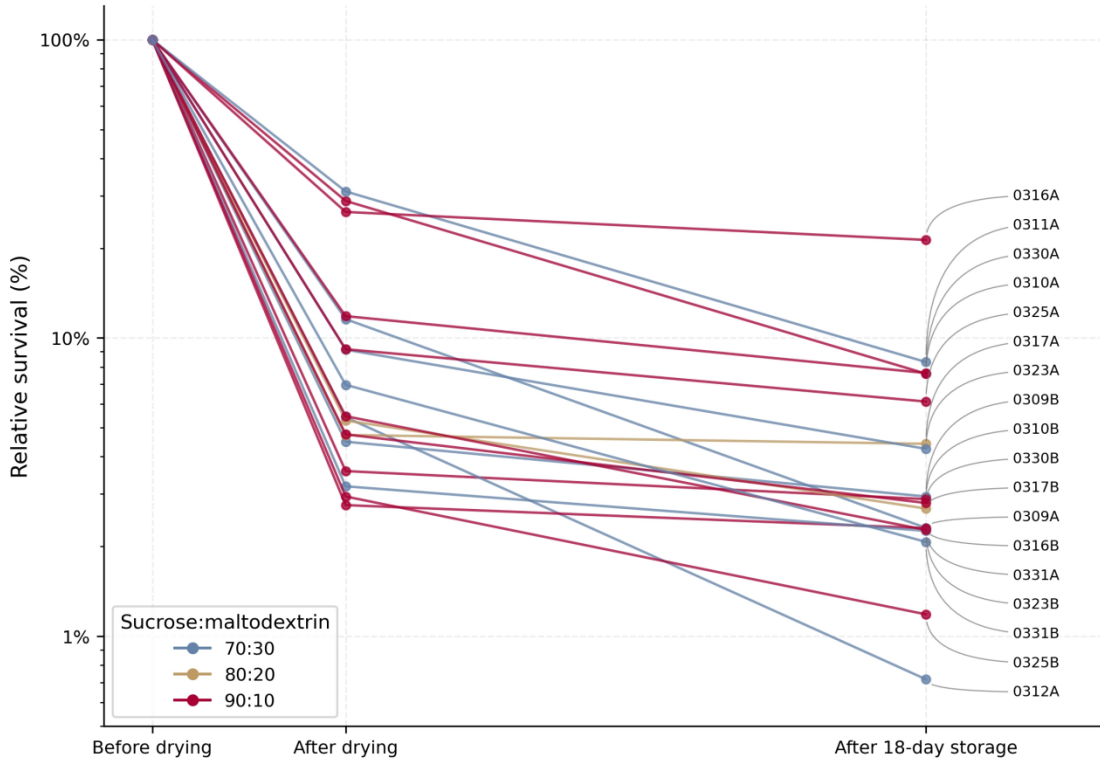

Figure 5. *Survival trajectory of VSD powders before drying, after vacuum spray drying, and after 18-day storage.*

The survival trajectories also suggested a formulation-dependent difference during the storage stage. From the after-drying stage to the after-storage stage, many of the 90:10 sucrose:maltodextrin batches showed a flatter trajectory, indicating a smaller relative decrease during storage compared with most 70:30 batches. This suggests that the 90:10 formulation may have been more favourable for preserving the remaining viable cells during short-term storage.

The relationship between after-drying viability and 18-day storage retention is shown in **Figure 6**. Overall, the two responses were not directly proportional across all batches. Some samples with low after-drying viability, such as 0317A, 0316B, and 0310B, retained a high fraction of the remaining viable cells during storage. In contrast, some samples with high after-drying viability, such as 0311A and 0310A, showed lower storage retention. Notably, batch 0316A combined both high after-drying viability and high storage retention, indicating favourable performance across both stages.

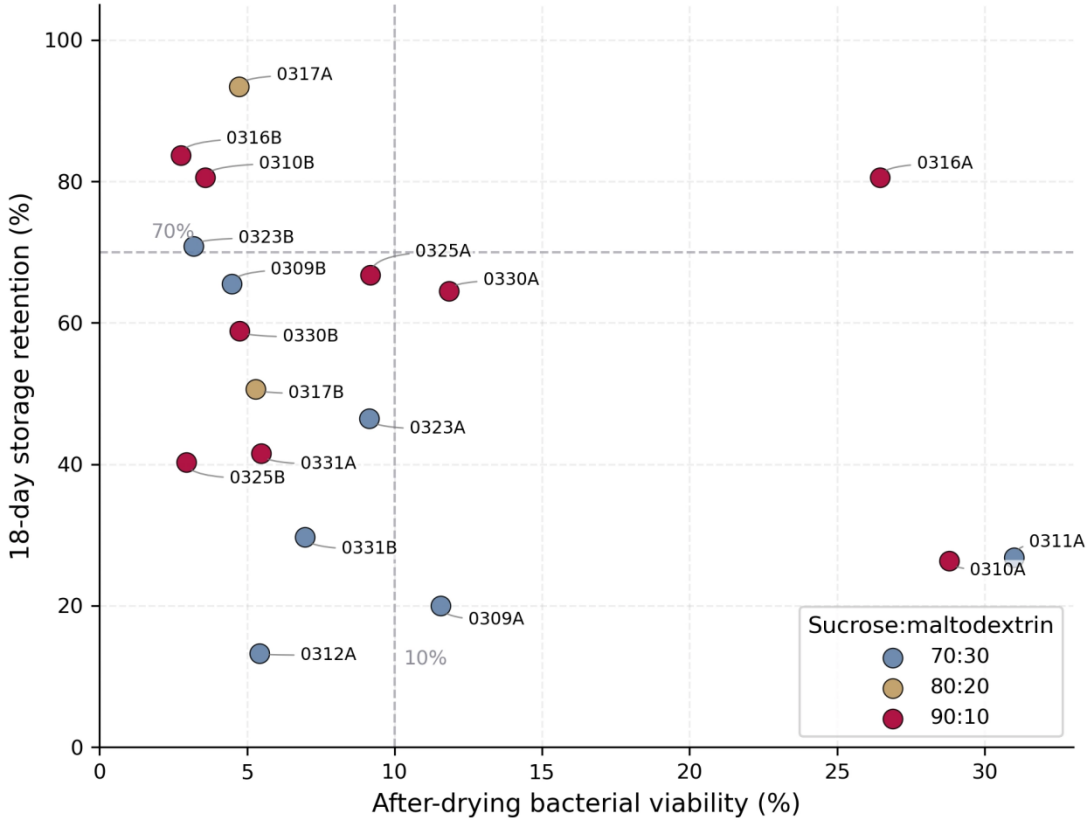

Figure 6. *Relationship between after-drying viability and 18-day storage retention of VSD powders. Dashed lines indicate reference levels of 10% after-drying viability and 70% storage retention.*

Together, these results show that final survival reflected the combined outcome of

survival during drying and retention during storage. Final survival therefore served as an important reference metric for comparing the overall biological performance of the batches, although the drying and storage stages were also interpreted separately.

3.6 Data-driven modelling of VSD outcomes

Data-driven modelling was used to further examine the relationships between process variables, formulation variables and selected VSD outcomes. Because the responses differed in their process meaning and available data structure, different modelling approaches were applied to different outcomes. Outlet temperature was modelled as an empirical process-state descriptor using the 2026 bacteria-containing dataset. Water activity was analysed using Ridge regression with the combined 2025–2026 VSD dataset to evaluate interpretable relationships between process and formulation variables and this physicochemical powder property. Bacterial viability was explored using Random Forest models because the survival response was more complex, non-linear and limited to the 2026 bacteria-containing experiments. The aim of this section was therefore not to establish a universal predictive model for VSD, but to identify interpretable trends and process–response relationships within the experimental window of this study.

3.6.1 Empirical modelling of outlet temperature

Outlet temperature was first examined as a process-state response of the VSD system. Among the interpretable candidate models, the model including chamber pressure, inlet temperature, sucrose content in solids and a pressure × inlet temperature interaction was selected because it retained a simple linear structure while giving consistently high internal validation performance. The selected model achieved a full-fit R^2 of approximately 0.993, LOOCV R^2 of approximately 0.984 and 4-fold CV R^2 of approximately 0.987, indicating that these variables captured most of the observed outlet-temperature variation within the tested experimental range.

The selected empirical model was:

$$Tout = 11.555940 - 0.106303P + 0.054148Tin - 0.031390S + 0.006675P \times Tin$$

where Tout is outlet temperature (℃), P is chamber pressure (kPa), T_{in} is inlet temperature (℃), and S is the sucrose content in the excipient solids (%).

The empirical model was consistent with the experimental trend shown in **Figure 1**. In general, outlet temperature increased with increasing inlet temperature and

chamber pressure within the tested VSD operating range. Although the main-effect coefficient of pressure was negative, the marginal effect of pressure, expressed as $-0.106303 + 0.006675 \times T_{in}$, remained positive across the tested inlet-temperature range of 70–130°C. Similarly, the marginal effect of inlet temperature, expressed as $0.054148 + 0.006675 \times P$, remained positive across the studied pressure range. The sucrose term was small and negative, suggesting that higher sucrose content was associated with a slightly lower outlet temperature in this empirical model.

Given its high internal validation performance, the selected model was used as an empirical outlet-temperature descriptor for downstream water activity and bacterial viability modelling. The equation should be interpreted as a local empirical relationship within the operating space explored in this study, rather than as a first-principles description of heat and mass transfer in the VSD process. Therefore, predictions outside the studied pressure, temperature and formulation range should be treated as exploratory and require further experimental validation.

3.6.2 Ridge regression modelling of water activity

Water activity was modelled using the combined 2025–2026 VSD dataset, including 45 observations with measured water activity. Two Ridge regression models were compared: a primary model using controllable process and formulation variables together with data-source indicators, and an auxiliary model that additionally included the empirical outlet-temperature descriptor from Section 3.6.1. The primary model achieved a 5-fold cross-validated R^2 of 0.789, RMSE of 0.0213 and MAE of 0.0183. Adding outlet temperature resulted in very similar performance, with $R^2 = 0.791$, RMSE = 0.0212 and MAE = 0.0185. Because outlet temperature was a derived process-state descriptor rather than an independent controllable input, and because its inclusion did not substantially improve cross-validated performance, the model without outlet temperature was retained as the primary interpretation model.

The standardized coefficients and cross-validated predictions of the primary Ridge model are shown in **Figure 7**. The coefficient plot indicated that inlet temperature and chamber pressure were the dominant negative contributors to predicted water activity, while the 2026 bacteria-containing data-source term and sucrose content showed positive coefficients. The predicted-versus-measured plot showed that the model captured the main variation in water activity, although deviations from the 1:1 line remained for some samples.

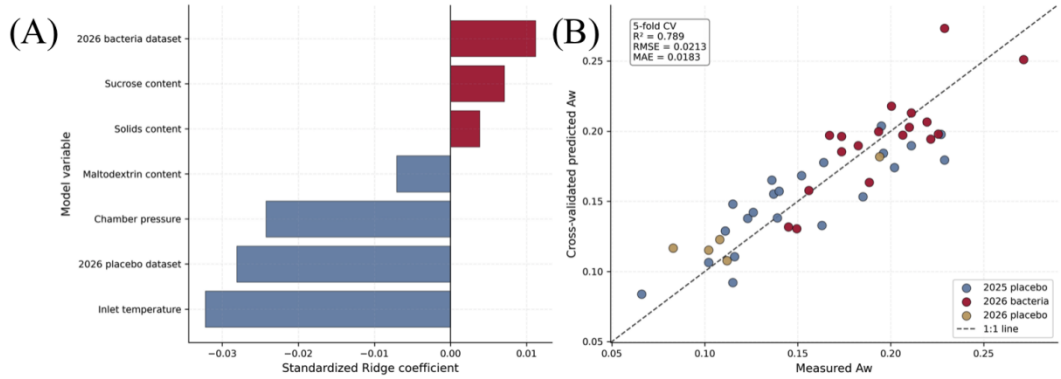

Figure 7. *Ridge regression modelling of water activity using the primary model without outlet temperature. (A) Standardized Ridge coefficients. Positive coefficients indicate association with higher predicted water activity, while negative coefficients indicate association with lower predicted water activity. (B) Cross-validated predicted versus measured water activity. The dashed line indicates the 1:1 line.*

In the primary model, higher inlet temperature and higher chamber pressure were associated with lower predicted water activity, consistent with more intensive drying conditions. The data-source terms suggested non-negligible systematic differences among the 2025 placebo, 2026 placebo, and 2026 bacteria-containing subsets, but should not be interpreted as direct causal factors. Sucrose and maltodextrin contents showed smaller and opposite standardized coefficients, reflecting their complementary formulation relationship.

3.6.3 Exploratory modelling of bacterial viability

The viability modelling workflow compared several survival-related responses, including after-drying bacterial viability, 18-day storage retention and final survival relative to before drying. Among these responses, after-drying viability showed the clearest cross-validated predictive signal. In contrast, storage retention and final survival were not robustly predicted under the current sample size, indicating that the available dataset was more informative for immediate drying survival than for longer-term stability outcomes. This was consistent with the structure of the available variables, which mainly described drying process conditions rather than storage-specific factors.

For after-drying viability, the best-performing models were obtained when process inputs were combined with the predicted outlet-temperature descriptor. Random Forest models outperformed the linear regression, Ridge regression and MLP alternatives in the baseline and grid-search workflows. The selected Random Forest model achieved a seed-averaged 4-fold CV R^2 of 0.682, RMSE of 5.01 percentage points, and MAE of 3.28 percentage points. The LOOCV result was similar, with R^2 =

0.706, RMSE = 4.82 percentage points and MAE = 3.05 percentage points. These results indicate that the selected feature set captured part of the non-linear variation in after-drying bacterial viability, although individual predictions remained uncertain because of the limited sample size.

The feature-importance results and cross-validated predictions are shown in **Figure 8**. The permutation-importance analysis indicated that the predicted outlet-temperature descriptor was the dominant explanatory feature in the Random Forest model, while the remaining process and formulation variables showed much smaller contributions. This suggests that, within the available 2026 bacteria-containing dataset, after-drying bacterial viability was mainly associated with the thermal process state represented by predicted outlet temperature. The result is consistent with the experimental observation that batches with lower outlet-temperature conditions tended to show higher after-drying viability.

The predicted-versus-measured plot showed that the model reproduced the general separation between low- and high-viability batches, although some individual deviations remained. Batch 0316A, which showed high after-drying viability, was positioned close to the 1:1 line. However, some high-viability batches, such as 0311A and 0310A, were underpredicted, whereas batch 0309A was overpredicted. This indicates that the model captured the overall separation between low- and high-viability batches, but individual batch-level predictions remained uncertain.

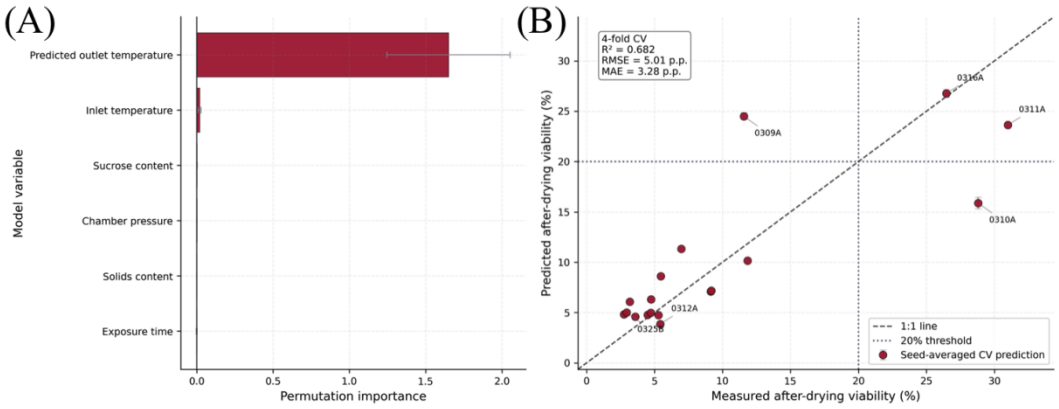

Figure 8. *Exploratory Random Forest modelling of after-drying bacterial viability. (A) Permutation feature importance of the selected Random Forest model. (B) Seed-averaged 4-fold cross-validated predicted versus measured viability. The dashed line indicates the 1:1 line; dotted lines indicate the 20% viability threshold.*

With only 18 bacteria-containing observations, the Random Forest model should be interpreted as an exploratory trend-level model rather than a validated predictive model. Nevertheless, the modelling results supported the experimental observation

that after-drying bacterial viability was strongly linked to the thermal process state represented by the predicted outlet-temperature descriptor.

3.7 Summary of feasible operating window

The feasible operating window was interpreted as the region where acceptable moisture-related powder properties and bacterial survival could be achieved simultaneously. Therefore, the operating window was not defined by a single response, but by the combined behaviour of water activity, after-drying viability, 18-day storage retention, and final survival. In this context, water activity was used as the main powder-drying indicator, while after-drying viability, storage retention and final survival were used to evaluate biological performance across drying and short-term storage. Outlet temperature was used as a process-state indicator to help interpret how drying intensity influenced these responses.

The relationship between outlet temperature and water activity is shown in **Figure 9**. Overall, batches with higher outlet temperature tended to show lower water activity, indicating that stronger drying conditions were generally associated with lower moisture availability in the dried powders. This trend was reflected by high-outlet-temperature batches such as 0309B and 0310B, which showed low water activity, whereas lower-outlet-temperature batches such as 0311A and 0316A showed higher water activity values above 0.22.

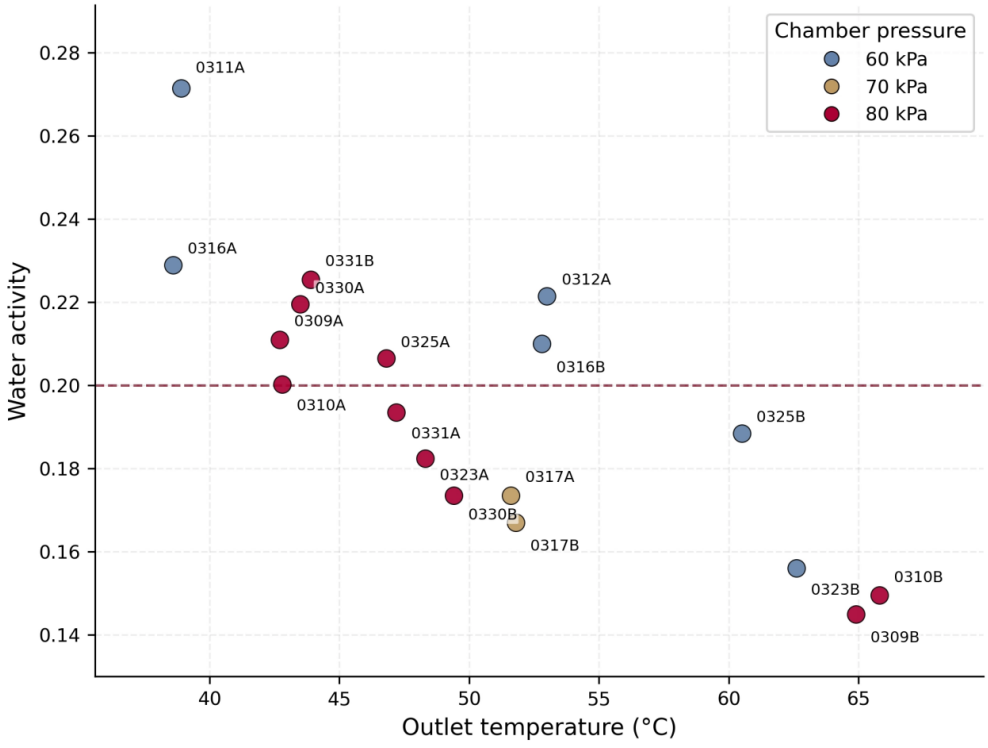

Figure 9. *Relationship between outlet temperature and water activity under different*

chamber pressure conditions. The dashed line indicates Aw = 0.20 as a reference level for low-water-activity powders.

The relationship between outlet temperature and bacterial survival outcomes is shown in **Figure 10**. Batches produced at lower outlet temperatures generally showed higher after-drying viability, whereas batches with higher outlet temperatures tended to show lower viability after drying. For example, 0311A, 0310A, and 0316A showed high after-drying viability values of approximately 31%, 29%, and 26%, respectively, and were associated with relatively low outlet temperatures. In contrast, batches such as 0309B, 0310B, 0323B, and 0325B showed low after-drying viability under higher outlet-temperature conditions. The final-survival plot showed a similar but less direct trend, as final survival also depended on retention during short-term storage.

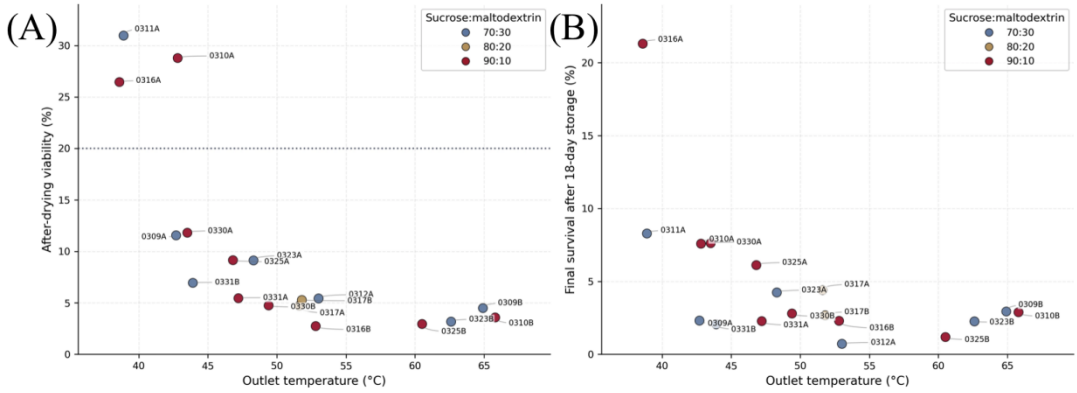

Figure 10. *Relationship between outlet temperature and bacterial survival outcomes. (A) Outlet temperature versus after-drying bacterial viability. (B) Outlet temperature versus final survival after 18-day storage.*

The relationship between water activity and bacterial survival outcomes is shown in **Figure 11**. This comparison was used to examine whether low water activity and high bacterial survival could be achieved simultaneously. The batches with higher viability were not among the lowest-water-activity samples in the dataset. Similarly, 0316A showed the highest final survival of approximately 21% after 18-day storage, while its water activity was 0.229. In contrast, the low-water-activity batches, such as 0309B and 0310B, did not show high after-drying viability, with values of approximately 4.5% and 3.6%, respectively. However, these low-water-activity batches still retained measurable bacterial survival after drying and storage, corresponding to approximately a 1–2 log reduction rather than complete loss of viable cells. This indicates that stronger drying reduced survival, but did not eliminate bacterial viability under the tested conditions.

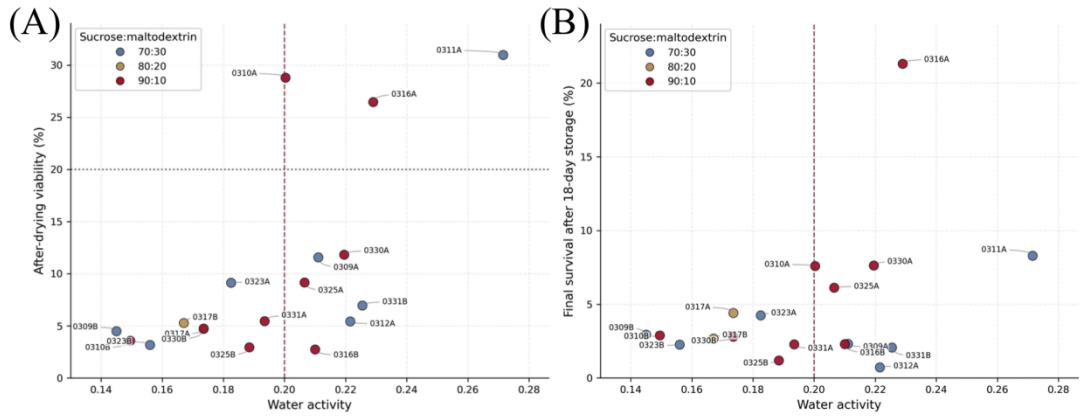

Figure 11. *Relationship between water activity and bacterial survival outcomes. (A) Water activity versus after-drying bacterial viability. (B) Water activity versus final survival after 18-day storage. The vertical dashed line indicates Aw = 0.20, and the horizontal dashed line in panel A indicates 20% after-drying viability.*

After considering the relationships among outlet temperature, water activity and bacterial survival, the biological performance of the batches was further compared across the drying and storage stages. The survival trajectory shown in **Figure 5** identified 0316A as the batch with the highest final survival after 18-day storage. **Figure 6** further showed that this batch was favourable because it combined relatively high after-drying viability of approximately 26% with high storage retention of approximately 81%. In contrast, 0311A and 0310A showed higher after-drying viability, approximately 31% and 29%, respectively, but lower storage retention of approximately 27% and 26%. Conversely, 0317A retained approximately 93% of the remaining viable cells during storage, but its after-drying viability was only approximately 4.7%.

Formulation composition was also considered in the operating-window interpretation. When final survival after 18-day storage was grouped by sucrose:maltodextrin ratio, the highest final survival was observed for batch 0316A in the 90:10 formulation group (**Figure 12**). Several other batches in the same formulation group, such as 0310A, 0330A, and 0325A, also showed relatively higher final survival. However, the 90:10 group also included batches with low final survival, such as 0316B and 0325B. This indicates that the 90:10 formulation may have contributed to favourable biological performance under selected process conditions, but formulation composition alone did not determine the final biological outcome.

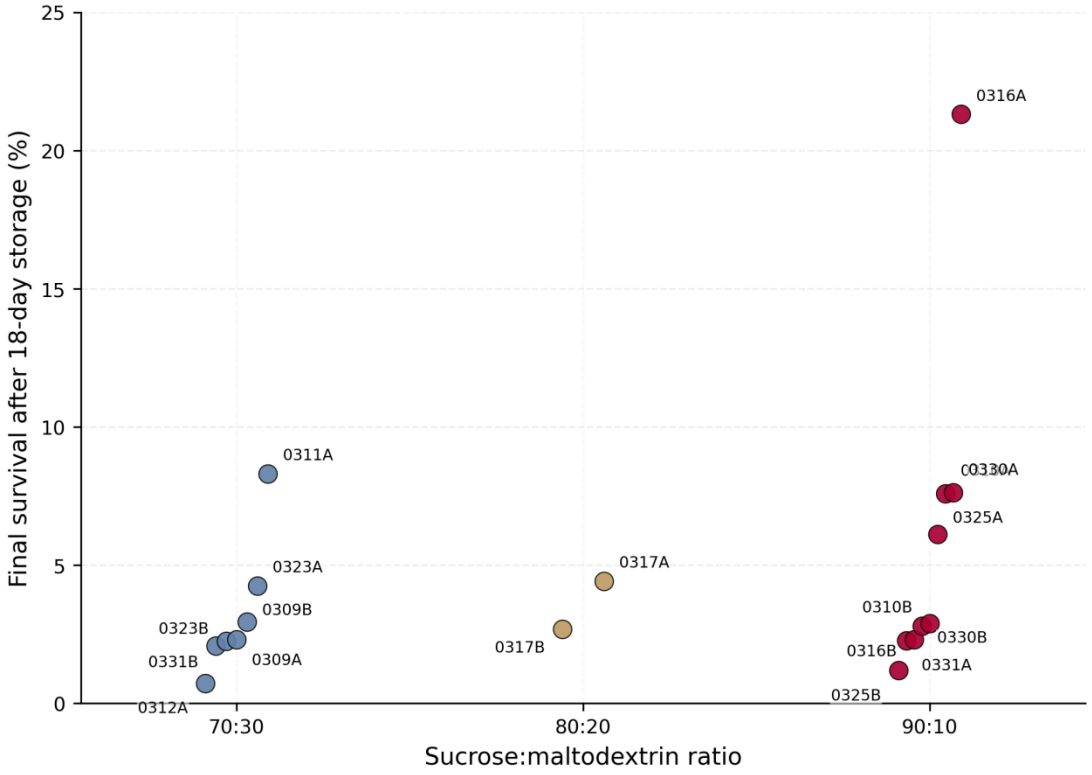

Figure 12. *Final survival after 18-day storage grouped by sucrose:maltodextrin formulation. Each point represents one VSD batch, labelled by batch ID.*

Batch 0316A was produced at 60 kPa chamber pressure, 80°C inlet temperature and a 90:10 sucrose:maltodextrin ratio. This condition resulted in an outlet temperature of 38.6°C, water activity of 0.229, after-drying viability of 26.5%, storage retention of 80.6%, and final survival of approximately 21%. Although its water activity was above the 0.20 reference level and was not the lowest among the tested powders, it provided the best overall balance between bacterial survival during drying and retention during short-term storage. It should therefore be interpreted as a promising candidate region for further optimisation rather than as a fully validated optimum.

4 Discussion

The present study should be interpreted as a transition from placebo-based VSD powder development to bacteria-containing process development. Previous work on the same YC-2000 VSD platform had already demonstrated the technical feasibility of producing sucrose–maltodextrin powders under reduced-pressure drying conditions, and showed that chamber pressure, inlet temperature, solids concentration, and formulation composition could affect outlet temperature, moisture content, water activity, and particle morphology. In particular, lower chamber pressure was associated with lower outlet temperature, while increasing inlet temperature improved drying efficiency and reduced moisture-related powder properties in the placebo system (Yang, 2025). These findings provided an important process and formulation basis for the present study. However, the introduction of *L. reuteri* changed the central development question from powder formation alone to the simultaneous achievement of acceptable powder quality and measurable bacterial survival after drying and short-term storage. Therefore, the present work extends the earlier placebo-based process understanding into a biologically constrained operating-window problem.

Building on this transition, the first process-level question was how the controllable VSD settings could be translated into a process-state description relevant to both powder formation and bacterial stress. In the present study, outlet temperature was used as the main process-state indicator linking drying intensity, powder dryness and bacterial survival. In the operating-window analysis, higher outlet-temperature conditions were generally associated with lower water activity, indicating more intensive drying, but they were also associated with lower after-drying viability. Conversely, higher after-drying viability was generally associated with lower outlet temperature but higher water activity. This interpretation is consistent with previous spray-drying work on lactic acid bacteria, where powder-side temperature and water activity were considered more relevant to viability loss than equipment setpoints alone (Martins et al., 2019).

The biological results also show that VSD caused partial but not complete loss of culturability. Even under less favourable conditions, viable cells were still recovered after drying and after 18 days of storage, indicating that the tested VSD conditions did not lead to complete inactivation of *L. reuteri*. Therefore, low-survival batches should not simply be treated as failed experiments, but as part of the process map defining the upper boundary of drying stress tolerated by the bacteria. In this context, batch

0316A occupied a Pareto-front position in the dataset. Although it was not the driest powder and its water activity was slightly above 0.20, it combined high after-drying viability with high storage retention.

This interpretation is consistent with the broader literature showing that water activity should be considered as an acceptable stability range rather than as a single universal optimum. In spray-dried *Lactococcus lactis*, an ideal Aw of approximately 0.198 was proposed for minimising immediate viability loss after drying, while storage studies have shown that higher Aw values, especially around 0.43, can accelerate probiotic viability loss during storage (Martins et al., 2019; Vesterlund et al., 2012). Therefore, the present results do not contradict the importance of low water activity. Rather, they suggest that within the relatively narrow and low Aw range obtained here, approximately 0.15–0.27, small differences in Aw were not sufficient to explain final biological performance alone. Overall, water activity and viability should be considered together, because neither the lowest Aw nor the highest immediate viability alone defined the most favourable operating condition.

This multi-response operating-window interpretation also shaped the modelling strategy used in this thesis. Because outlet temperature, water activity and bacterial viability represented different levels of process response, the aim of the data-driven analysis was not to build a single multi-output black-box predictor, but to extract interpretable process–response relationships from a small experimental dataset. Previous spray-drying studies have used artificial neural network (ANN) models for multi-output prediction of process and powder-quality attributes, such as outlet temperature, residual moisture, particle size, and yield (Chegini et al., 2008; Ming et al., 2021). These studies show that neural-network models can be useful for capturing non-linear relationships when sufficient data are available and the prediction targets are well defined. By contrast, the present bacteria-containing VSD dataset was limited in size, making an ANN-based approach less suitable for the present interpretive aim.

Accordingly, model complexity was adjusted according to the nature of each response. Outlet temperature was modelled using a simple empirical linear framework, reflecting its relatively deterministic relationship with chamber pressure and inlet temperature. Water activity was treated as a physicochemical powder-state response and analysed using Ridge regression, which provided a regularised and interpretable model suitable for limited data. In contrast, bacterial viability was treated as a more complex biological response, because survival after drying can be influenced by interacting stress mechanisms, including thermal exposure, dehydration, osmotic

stress, formulation-dependent protection and cell-to-cell heterogeneity. For this reason, Random Forest modelling was used exploratorily for after-drying viability, and the resulting feature-importance analysis supported the experimental observation that viability was strongly linked to the outlet-temperature-related drying state. MLP models were also evaluated during model screening and grid search, but their cross-validated performance was less robust than that of Random Forest models under the current sample size.

A useful benchmark for interpreting the present VSD results is freeze-drying of the same probiotic strain. Freeze-drying remains the established reference process for sensitive probiotic and biologic formulations because it enables water removal at low product temperatures and can achieve high immediate survival (Sharma et al., 2021). In Bai's work on freeze-dried *L. reuteri* pellets, favourable pellet conditions gave substantially higher drying survival than the present VSD process (Bai, 2024), indicating that the current low-temperature VSD protocol did not yet reach the cell-preservation level of optimised freeze-drying. However, this comparison should be interpreted with caution because the bacteria used in the present study had already been commercially freeze-dried, rehydrated, and washed before VSD processing, whereas the freeze-drying benchmark was based on freshly prepared cells. Therefore, the initial physiological state of the bacteria was different, which limits direct comparison of survival levels between the two studies. The water activity values obtained in the present VSD powders, including 0.229 for the best-performing batch 0316A, further indicate that moisture-related stability was not fully optimised and should be addressed in future long-term storage studies. Nevertheless, the present VSD results show that the process did not lead to complete inactivation: viable cells were recovered after drying and after 18-day storage across the tested batches, and some conditions retained appreciable survival after both stages.

Future work should first repeat and refine the 0316A condition to assess whether this promising process–formulation region is reproducible. Longer storage studies are also needed, extending beyond the current 18-day period and including repeated measurements of CFU, water activity and, preferably, vitality-related indicators at each storage time point. This is important because water activity may change during storage as water redistributes within the dried matrix, and initial water activity may not fully describe the powder state during later storage. In addition, future studies should consider using freshly cultivated cells and duplicate experiments to reduce the uncertainty associated with commercial freeze-dried starting material. Further optimisation could also examine bacterial concentration, strain-dependent robustness,

and additional vitality assays. With a larger and more systematic dataset, data-driven modelling could be extended from exploratory trend identification toward more robust process–formulation–stability modelling.

From an industrial process-development perspective, these results support the positioning of VSD as a potential alternative drying route at this stage. Freeze-drying provides stronger immediate cell preservation, but it is also associated with long processing times, high energy demand, and higher production costs, whereas spray-drying-based processes offer advantages in speed, scalability, and continuous processing potential (Wahab et al., 2025). Therefore, the relevance of VSD should not be judged only by whether the current survival level matches optimised freeze-drying. If the remaining viability loss can be reduced or managed through process optimisation, formulation improvement, tighter control of water activity and storage conditions, or sufficient starting cell concentration, VSD may still offer a useful low-temperature drying route for LBP manufacturing. The value of the present study is therefore not to claim that VSD is already superior to freeze-drying, but to establish an initial bacteria-containing VSD operating map, identify a promising process–formulation region, and demonstrate how data-driven analysis can support further optimisation of LBP drying.

5 Conclusion

This thesis demonstrated that low-temperature vacuum spray drying can produce bacteria-containing *Limosilactobacillus reuteri* DSM 17938 powders with measurable viability after drying and short-term room-temperature storage. Within the tested experimental window, the main process challenge was the trade-off between drying intensity, moisture-related powder properties, and bacterial survival. Higher outlet-temperature conditions generally reduced water activity but were associated with greater viability loss, whereas milder drying conditions improved after-drying viability but did not always produce the lowest water activity. Among the tested batches, 0316A represented the most promising candidate region, combining relatively high after-drying viability, high 18-day storage retention, and the highest final survival, although the reproducibility and robustness of this condition still require further validation. The data-driven analysis of outlet temperature, water activity and bacterial viability supported the use of outlet temperature as a useful process-state descriptor and indicated that after-drying viability was strongly linked to the outlet-temperature-related drying state, while the modelling results remained exploratory because of the limited number of bacteria-containing experiments. Overall, this study supports VSD as a promising low-temperature drying route for probiotic and LBP formulation development, while also highlighting the need for further process optimisation, formulation improvement, tighter control of water activity, and controlled storage studies to improve viability preservation and assess its practical applicability.

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Appendix A. Full experimental dataset

Table A1. Process and powder properties.

Batch ID	Pressure (kPa)	Solids (% w/w)	Sucrose in solids (%)	Maltodextrin in solids (%)	T_{in} (°C)	Feed holding time (min)	Exposure time (min)	Tout (°C)	Aw	Moisture content (%)	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	Span	Powder recovery (%)
0309A	80	30	70	30	70	7	10	42.7	0.211	4.77	6.667	19.048	234.021	11.936	50
0309B	80	30	70	30	110	9	8	64.9	0.145	3.59	4.755	13.557	29.604	1.833	40
0310A	80	30	90	10	70	8	9	42.8	0.200	4.95	4.901	15.903	55.127	3.158	30
0310B	80	30	90	10	110	8	10	65.8	0.150	4.03	4.138	13.209	43.655	2.992	33
0311A	60	30	70	30	80	9	9	38.9	0.272	6.26	4.649	12.668	31.085	2.087	30
0312A	60	30	70	30	110	10	9	53.0	0.222	5.14	4.234	11.486	27.61	2.035	43
0316A	60	30	90	10	80	7	8	38.6	0.229	5.54	5.548	18.061	42.823	2.064	23
0316B	60	30	90	10	110	8	9	52.8	0.210	5.02	5.01	12.971	28.414	1.804	20
0317A	70	30	80	20	95	8	9	51.6	0.174	4.36	4.829	14.038	31.664	1.912	47
0317B	70	30	80	20	95	7	9	51.8	0.167	4.22	4.072	11.62	31.193	2.334	43
0323A	80	30	70	30	80	7	9	48.3	0.183	4.65	5.531	14.186	34.653	2.053	53
0323B	60	30	70	30	130	9	9	62.6	0.156	4.45	4.886	11.052	23.419	1.677	37
0325A	80	30	90	10	80	8	9	46.8	0.207	5.00	6.154	15.396	32.382	1.704	43
0325B	60	30	90	10	130	8	9	60.5	0.189	4.95	4.136	11.939	29.195	2.099	30
0330A	80	30	90	10	75	8	10	43.5	0.220	5.55	4.934	11.504	25.653	1.801	40
0330B	80	30	90	10	85	8	9	49.4	0.174	4.58	4.316	10.826	24.276	1.844	40
0331A	80	30	90	10	80	8	9	47.2	0.194	3.86	4.794	11.57	26.15	1.846	57
0331B	80	30	70	30	75	8	8	43.9	0.226	5.44	3.994	8.454	19.266	1.806	43

Table A2. CFU and survival metrics.

Batch ID	Before-drying CFU/g or CFU/mL	After-drying CFU/g	After-storage CFU/g	After-drying viability (%)	18-day storage retention (%)	Final survival (%)
0309A	5.02E+10	5.80E+09	1.16E+09	11.6	20.0	2.3
0309B	3.82E+10	1.71E+09	1.12E+09	4.5	65.5	2.9
0310A	3.28E+10	9.46E+09	2.49E+09	28.8	26.3	7.6
0310B	3.41E+10	1.22E+09	9.83E+08	3.6	80.5	2.9
0311A	1.83E+10	5.66E+09	1.52E+09	31.0	26.8	8.3
0312A	3.45E+10	1.87E+09	2.47E+08	5.4	13.2	0.7
0316A	2.96E+10	7.82E+09	6.30E+09	26.5	80.6	21.3
0316B	4.79E+10	1.32E+09	1.10E+09	2.8	83.7	2.3
0317A	4.58E+10	2.17E+09	2.02E+09	4.7	93.4	4.4
0317B	4.90E+10	2.59E+09	1.31E+09	5.3	50.7	2.7
0323A	3.85E+10	3.52E+09	1.64E+09	9.1	46.5	4.2
0323B	4.10E+10	1.30E+09	9.24E+08	3.2	70.9	2.3
0325A	3.69E+10	3.39E+09	2.26E+09	9.2	66.8	6.1
0325B	3.74E+10	1.10E+09	4.43E+08	2.9	40.3	1.2
0330A	3.29E+10	3.89E+09	2.51E+09	11.8	64.5	7.6
0330B	4.74E+10	2.25E+09	1.32E+09	4.7	58.9	2.8
0331A	4.46E+10	2.44E+09	1.01E+09	5.5	41.6	2.3
0331B	4.18E+10	2.91E+09	8.65E+08	7.0	29.7	2.1