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Research article

Improving probiotic preservation through phycocyanin enriched skim milk as a protective drying medium

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Abstract

The viability and functionality of probiotics are strongly influenced by environmental stressors encountered during processing and storage. This study aimed to evaluate the effects of phycocyanin supplementation and drying in skim milk, followed by vacuum desiccation at 50 °C, on three probiotic strains : *Limosilactobacillus reuteri*, *Lactiplantibacillus plantarum*, and *Lacticaseibacillus casei*. Probiotic viability, metabolic activity, and surface properties were monitored over 12 months of storage at room temperature, refrigeration, and freezing, with assessments performed every three months.

Results showed that phycocyanin supplementation markedly improved survival, particularly at room temperature, with increases of up to 2 log CFU/g. Drying in skim milk further enhanced stability and promoted biofilm-forming ability. Additionally, phycocyanin positively affected metabolic activity and cell surface interactions.

Overall, these findings demonstrate that combining phycocyanin supplementation with vacuum desiccation in skim milk represents an effective approach for enhancing long-term probiotic preservation under diverse storage conditions.

Keywords: Probiotics, phycocyanin, skim milk, hydrophobicity, adhesion, viability.

Introduction

The World Health Organization defines probiotics as “ live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Mack 2005; Malli et al., 2019). These microorganisms have long been recognized as essential biological agents and valuable commercial targets owing to their diverse health-promoting properties (Hamad et al., 2022; Latif et al., 2023). Most probiotic strains used as probiotics belong to the genera *Lactobacillus* and *Bifidobacterium.* Other microbial species that can also serve as probiotics include *Bacillus*, *Streptococcus*, *Enterococcus*, and *Saccharomyces* (Sarita et al., 2025). They can be consumed either by incorporating them into foods or drinks in the form of dairy or non-dairy foodstuffs or as supplements (Fernandez et Marette 2017). The approximate consumption of 10^9 colony-forming units (CFU)/day has been revealed as an effective dose (Hill et al., 2014). Maintaining the viability and metabolic activity of these microorganisms in the gastrointestinal tract is crucial for their beneficial effects, which include modulation of gut microbiota (Kim et al., 2021;Nyanzi et al., 2021), enhancement of immune function (Shamekhi et al. 2020), and prevention of gastrointestinal infections (Milner et al., 2021).

The viability and stability of probiotics are critical factors influencing their effectiveness in different applications, including food and pharmaceutical products (Terpou et al., 2019). However, preserving probiotic viability during manufacturing, storage, and distribution remains challenging, as these microorganisms are highly sensitive to environmental stressors such as temperature fluctuations, redox potential, humidity, and desiccation (Klinmalai et al., 2025). Among the different preservation techniques, drying has emerged as one of the most effective and widely recommended methods for ensuring the long-term stability and viability of

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probiotics. This process reduces the water activity of the product, thereby limiting microbial metabolism and degradation reactions, while facilitating easier handling, transportation, and incorporation into various formulations (Noufeu et al., 2025). Nevertheless, the survival of probiotic strains during the drying process is strongly influenced by the protective matrix used. Dairy-based materials, particularly regular and low-fat milk and their derivatives, are frequently employed as protective matrices due to their rich nutrient composition, which not only supports the metabolic activity of probiotic cells but also shields them from environmental stress during processing and storage (Wasana et al., 2025). The survival of probiotics in skim milk has not been thoroughly studied, even though whole and reduced fat milk has been the subject of much research in the preparation of probiotics. Skim milk provides a protein-rich environment without the complicating presence of lipids, which can alter drying conditions and, ultimately, the stability of probiotics (Kil et al., 2020). Incorporating suitable protective agents during drying, an essential step in the long-term preservation of microorganisms, has also been shown to significantly enhance probiotic survival.

In recent years, there has been renewed interest in natural protective agents, particularly compounds derived from microorganisms. One notable example is phycocyanin, a biliprotein obtained from cyanobacteria such as *Spirulina*, which is known for its antioxidant and protective properties. This water-soluble, non-toxic, blue colored photosynthetic pigment has been applied in the food, cosmetic, and pharmaceutical industries (De Morais et al., 2018). It exhibits a wide range of biological activities such as antioxidant, anti-inflammatory and cytoprotective properties (Martelli et al. 2014), and is therefore an interesting candidate to enhance stability of probiotics (Valikboni et al., 2024; Chakroun et al., 2023). Subsequently, considering phycocyanin as naturally derived compound which contributes to cellular protection, it is an attractive substitute for synthetic stabilizers used for probiotic preservation. The phycocyanin-probiotic matrix is promising because phycocyanin has antioxidant properties that may serve to reduce oxidative stress and enhance probiotic cell survival during production and storage conditions, and potentially protect against stressors such as heat and desiccation, which have been problematic for probiotics in pharmaceutical applications and food (Gorgich et al., 2020). Studies on the synergistic effect of phycocyanin and probiotics suggest that phycocyanin could work as a natural stabilizer for increasing the applicability and survival scale of probiotics during both production and delivery under hostile conditions. This offers an interesting strategy to improve probiotics formulations, particularly those with prolonged shelf-life and/or hostile environment as in the case of functional food or food supplements.

Considering these aspects, the novelty of this study lies in the combination of phycocyanin with skim milk as a protective matrix for probiotic drying. Phycocyanin, due to its antioxidant and cytoprotective properties, is expected to enhance probiotic survival more effectively than other natural protectants. Its use provides a natural alternative to synthetic stabilizers, potentially improving cell viability, metabolic activity, and shelf-life during storage and rehydration (Valikboni et al., 2024; De Morais et al., 2018; Chakroun et al., 2023). This innovative approach allows a clear evaluation of the protective effects of phycocyanin within a protein-rich, lipid-free matrix, highlighting its potential application in functional foods and probiotic supplements.

This research offers insights into these aspects together with the integrated effects of drying process, skim milk as a protective matrix, and phycocyanin on probiotics viability, cell stability, metabolic activity and production of lactic acid during storage and rehydration

Material and methods

Culture and Preparation of Probiotics

Three probiotic strains were used in this study, included *Limosilactobacillus reuteri* (OL468126.1) and two reference strains, *Lactiplantibacillus plantarum* ATCC 8014 and *Lacticaseibacillus casei* ATCC 334. These strains were chosen for their probiotic potential and technological significance. The bacterial cultures were grown anaerobically in de Man, Rogosa, and Sharpe (MRS) broth (MRS; Difco, BD Diagnostic Systems, Sparks, MD, USA; Catalog No. MHA00MRS2) at 37 °C for at least 48 h.

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Following incubation, bacterial cells were harvested by centrifugation at 10,000 × g for 10 min at 4°C to minimize heat stress. The resulting pellets were washed twice with sterile saline solution and subsequently resuspended in 250 mL of sterile skim milk (10 % w/v) to obtain a final cell concentration of approximately 1 x 10^9 CFU/mL, as determined from the original culture by plate counting. All procedures were performed under aseptic conditions to maintain culture viability and prevent contamination.

Source and concentration of C-phycocyanin extract

C-phycocyanin extract (8 mg/mL, food grade) was obtained from Bioalgae Tunisia Society (www.alguespiruline.net) and used as provided without further purification.

Drying process of probiotics

Probiotic bacterial cultures were harvested and resuspended in 250 mL of skim milk (10 % w/v) at a final concentration of approximately 1 x 10^9 CFU/mL. The suspension was dried under vacuum in a glass desiccator at 50°C for 48 h to account for the high moisture content (~90). In selected experimental setups, C-phycocyanin was added to the skim milk at different concentrations (1%, 3%, 5%, and 7% v/v) (data not shown) to evaluate its potential protective effect on probiotic viability. Based on preliminary experiments, 5% (v/v) was identified as the optimal concentration, providing the best balance between enhancing probiotic survival and maintaining the technological properties of the formulation. Drying was considered complete when a constant weight was reached.

Rehydration of probiotics after drying

Following the drying process, the probiotics were rehydrated in sterile phosphate-buffered saline (PBS) at a 1 :10 (w/v) ratio (1g of dried probiotic powder in 10 mL PBS) to minimize any potential influence from components of complex culture media. Rehydration was performed for 30 minutes at room temperature (25°C) with gentle agitation to ensure complete dissolution of the probiotic particles. After rehydration, the samples were incubated at 37°C under anaerobic conditions for 48 hours to allow for bacterial recovery prior to subsequent viability assays, ensuring that the probiotics were fully resuscitated before further analysis.

Estimation of probiotic viability and shelf-life stability

The viability of probiotics was determined as colony forming units (CFU) using the plate count technique after drying. Subsequently, the strains were placed in one of three environments: room temperature (25°C), refrigerated (4°C), or frozen (-20°C). Probiotic viability was tracked long-term, and samples were taken every three months until 12 months passed. At each point, **three independent replicate samples** were analyzed, and the mean CFU values ± standard deviations were calculated. Results were compared against the initial CFU measured immediately after drying to assess the effectiveness of the storage conditions on the stability and functionality of the probiotics (Mahmoodian et al., 2024)**.**

Probiotic surface adhesion assay to solvent

The cell surface properties of the probiotics were assessed using the Bacterial Adhesion to Solvent (BATS) assay, as described by Kos et al. (Kos et al., 2003), with some modifications. The cultured probiotic bacterial cells were resuspended in PBS to match the bacterial concentration to a standard 10^8 CFU/ml concentration (Ho). The cell suspensions (3 ml each) were vortexed with 1 ml of hexadecane, chloroform, or ethyl acetate for 1 minute followed by 5 minutes allowed to stand to permit separation of the aqueous phase. H_t represents the number of viable bacterial cells (CFU mL^{-1}) remaining in the aqueous phase after the adhesion assay, and H_o represents the initial number of viable bacterial cells (CFU mL^{-1}) in the original bacterial suspension before the assay. The percentage of adhesion is calculated using the equation:

$$\textbf{Adhesion \%} = [1 - (\mathbf{H_t}) / (\mathbf{H_o})] \times 100$$

Based on their adhesion percentage, the strains were classified into three categories: high hydrophobicity (≥70%); moderate hydrophobicity (50–70%); and low hydrophobicity (<50%). All tests were

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performed in biological triplicate**,** using three independent cultures for each strain. The BATS assay was performed prior to vacuum drying and subsequently after vacuum drying to determine the impact of the drying treatment on the surface properties of probiotics, in the presence and absence of 5% (v/v) phycocyanin.

Determination of viability of probiotics in biofilms

To determine the viability of microbial cells within the biofilm, probiotic cultures were first rehydrated and resuspended to a final concentration of approximately 1 x 10^9 CFU/mL in sterile MRS broth. Biofilms were formed by inoculating 100 µL of this bacterial suspension into the wells of a sterile 96 well microtiter plate, followed by incubation at 37°C under anaerobic conditions for 48 hours to allow for biofilm development.

After biofilm formation, the planktonic cells were removed, and 150 µL of PBS along with 50 µL of MTT solution (0.3%) were added to each well. Plates were incubated for 2 hours at 37°C. Subsequently, the MTT solution was discarded, and 150 µL of DMSO and 25 µL of glycine buffer (0.1 M, pH 10.2) were added to dissolve the formazan crystals (Kairo et al., 1999). The plates were then incubated for 15 minutes at room temperature. Absorbance was recorded at 550 nm using a Microplate Reader Model 550 (Bio-Rad) to determine cell viability within the biofilms.

Assessment of Metabolic Activity: Lactic Acid Production

Lactic acid production, a crucial metabolic byproduct, was measured to evaluate the metabolic activity of the dried probiotic strains after rehydration. The rehydrated probiotic strains were cultivated in MRS broth at 37°C in anaerobic conditions for 24 to 48 hours. After incubation, 10 mL of the culture supernatant was collected and titrated with 0.1 N sodium hydroxide (NaOH) solution, using phenolphthalein as an indicator, according to established acid- base titration protocols (American Dairy Products Institute, 2023). The volume of NaOH required to neutralize the lactic acid will be used to calculate its concentration in the medium.

In parallel, bacterial viability was assessed by plating aliquots of the culture on MRS agar and performing colony count (CFU), followed by colony counting (CFU). This combined approach allowed a comprehensive understanding of the relationship between bacterial viability and their capacity to produce beneficial metabolites, which is essential for assessing the functional performance of probiotics after drying and rehydration.

Statistical analysis

The results are expressed as mean ± standard deviation. Data were statistically analyzed by one-way analysis of variance to determine differences among groups and Tukey test as a post hoc. All the statistical analyses were conducted using Statistical Package for Social Science (version 19.0, SPSS for Windows, USA) and differences were considered statistically significant when $p < 0.05$.

Results

Impact of storage temperature and phycocyanin on probiotic survival over 12 months

At room temperature, the viability of probiotics declined markedly in the absence of phycocyanin. *Lactobacillus reuteri* decreased from 5.12 log CFU/g at T0 to undetectable levels after 12 months of storage (Figure 1a). In contrast, samples supplemented with phycocyanin retained detectable viability, with *L. reuteri* maintaining approximately 1.47 log CFU/g after 12 months (Figure 1b).

Similar trends were observed for *L. plantarum* and *L. casei*, for which phycocyanin-treated samples showed 1.5–2.0 log CFU/g higher viability compared to untreated samples after 12 months at room temperature.

Under refrigerated conditions (4 °C), all strains exhibited reduced viability loss. After 12 months, *L. plantarum* maintained 3.5 log CFU/g with phycocyanin compared to 3.0 log CFU/g without supplementation.

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Freezing at −20 °C resulted in the highest preservation of probiotic viability, with only minimal reductions over the storage period. In all strains, phycocyanin-treated samples retained more than 2 log CFU/g after 12 months.

Production of lactic acid before and after drying with or without phycocyanin

As shown in Table (1), drying significantly reduced lactic acid production in all tested strains. However, samples dried in skim milk supplemented with 5% phycocyanin exhibited higher lactic acid production compared to samples dried without phycocyanin.

For *L. reuteri*, lactic acid production after drying reached 82.1% of the initial level (1.38 ± 0.03 g/L) in the presence of phycocyanin, compared to 59.4% (0.95 ± 0.04 g/L) in its absence. Similarly, *L. plantarum* retained 83.33% of its original lactic acid production (1.90 ± 0.04 g/L) with phycocyanin, whereas retention decreased to 60% (1.32 ± 0.05 g/L) without supplementation. *L. casei* followed a comparable pattern.

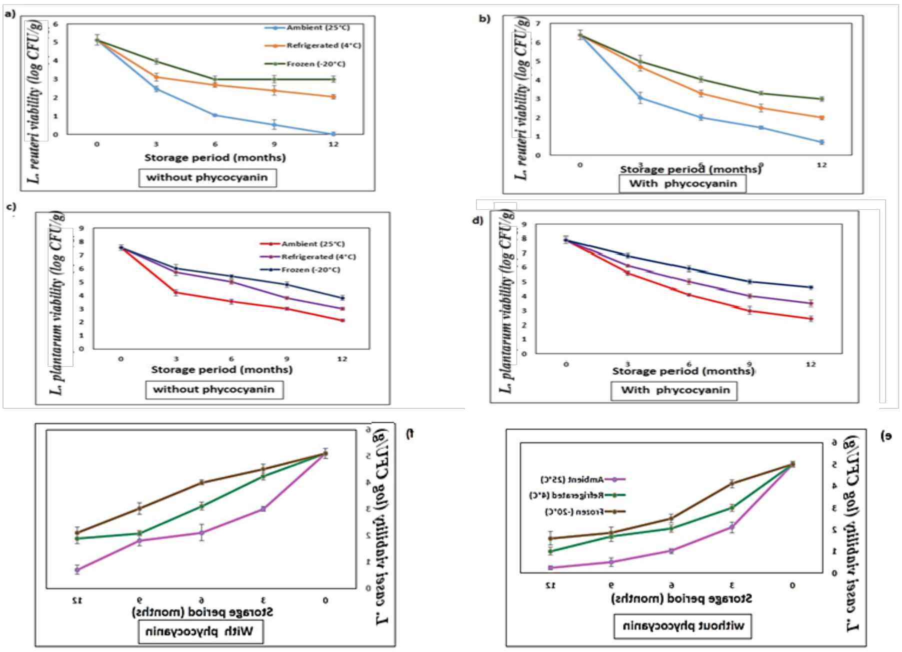

Figure (1): Viability of *L. reuteri* (a,b), *L. plantarum* (c,d), and *L. casei* (e,f) under different storage conditions (Room Temperature, 4°C, and -20°C), with and without phycocyanin, over a period of 12 months.

Table (1): Lactic acid production (g/L) before and after drying

Strain	Condition	Before Drying (g/L)	After Drying (g/L)	% Metabolic Retention
L. reuteri	Without phycocyanin	1.60 ± 0.05^{a}	0.95 ± 0.04^{c}	59.4%
L. reuteri	With phycocyanin	1.68 ± 0.04^{a}	1.38 ± 0.03^{a}	82.1%
L. plantarum	Without phycocyanin	2.20 ± 0.06^{b}	1.32 ± 0.05^{b}	60.0%
L. plantarum	With phycocyanin	2.28 ± 0.04^{b}	1.90 ± 0.04^{a}	83.3%
L. casei	Without phycocyanin	1.85 ± 0.03^{c}	1.00 ± 0.03^{c}	54.1%
L. casei	With phycocyanin	1.92 ± 0.05^{c}	1.56 ± 0.04^{a}	81.3%

[†]Statistical analysis denoted by the superscripts ^{a}, ^{b}, and ^{c} indicates that values within the same column sharing the same letter are not significantly different ($p > 0.05$). Horizontal comparisons between ‘Without phycocyanin’ and ‘With phycocyanin’ for the same strain at a given time point were also performed; statistically significant differences are reflected by differing letters in the “After Drying” column, showing the protective effect of phycocyanin on lactic acid production.

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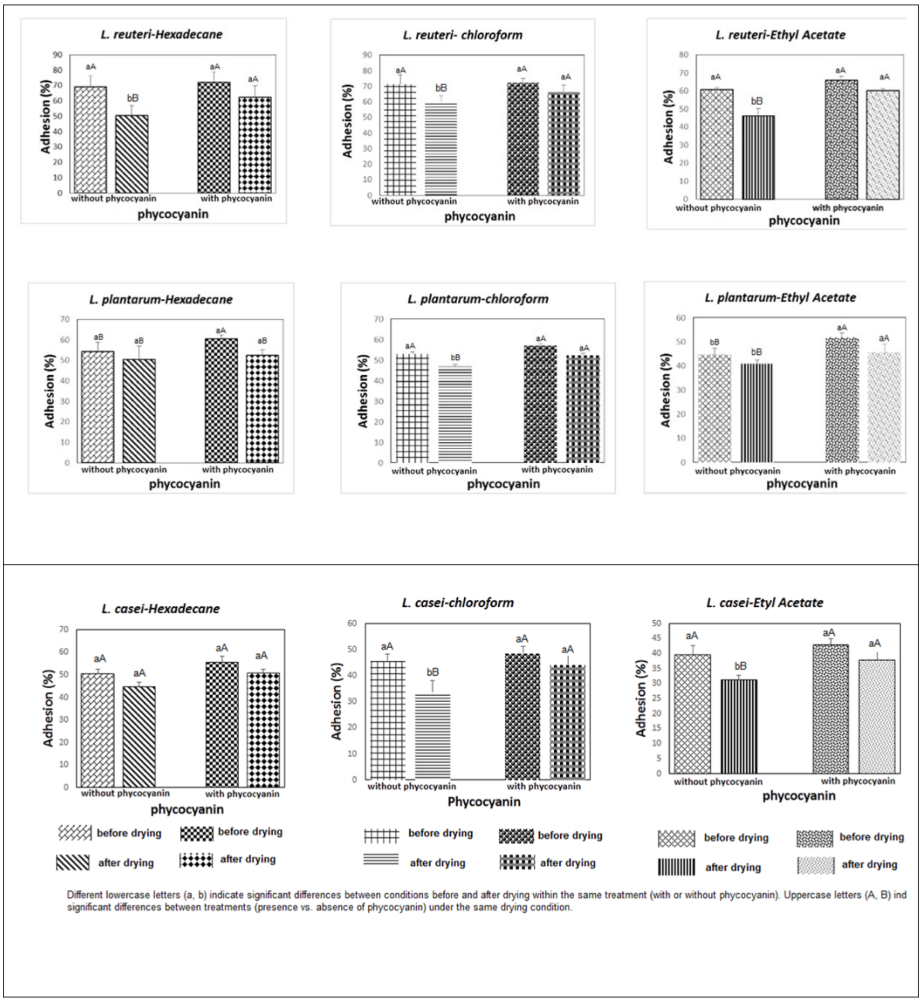
Impact of vacuum drying and phycocyanin on probiotic adhesion to solvents

Surface hydrophobicity was assessed using the Bacterial Adhesion to Solvents (BATS) method (Figure 2). Vacuum drying resulted in reduced adhesion to all tested solvents (hexadecane, chloroform, and ethyl acetate) for the three *Lactobacillus* strains, indicating a decrease in surface hydrophobicity.

For *L. reuteri*, adhesion to hexadecane decreased from 69.14% to 50.34% without phycocyanin and from 72.24% to 62.35% with phycocyanin. In *L. plantarum*, adhesion to hexadecane decreased from 60.56% to 52.46% in phycocyanin-treated samples, compared to a reduction from 54.40% to 50.34% in untreated samples. A similar trend was observed for *L. casei*. Across all strains, adhesion was highest toward hexadecane, followed by chloroform and ethyl acetate.



Different lowercase letters (a, b) indicate significant differences between conditions before and after drying within the same treatment (with or without phycocyanin). Uppercase letters (A, B) ind significant differences between treatments (presence vs. absence of phycocyanin) under the same drying condition.

Figure (2): Effect of drying and phycocyanin presence on the adhesion properties of *L. reuteri*, *L. plantarum*, and *L. casei* strains to hexadecane, chloroform, and ethyl acetate solvents.

Biofilm viability of *Lactobacillus* strains after drying and phycocyanin supplementation

Biofilm-associated cell viability, assessed by the MTT assay, decreased significantly after drying in all strains. For example, *L. reuteri* biofilm viability declined from 0.87 ± 0.05 to 0.62 ± 0.04 ($p < 0.05$). Supplementation with 5% phycocyanin significantly mitigated this reduction.

Among the strains tested, *L. plantarum* exhibited the highest biofilm viability and lactic acid retention following drying, particularly in the presence of phycocyanin.

Table (2): Viability of Probiotic Strains in Biofilm (MTT Assay)

Strain	Condition	Absorbance (Mean ± SD)
L. reuteri	Before drying, without phycocyanin	0.87^{b} ± 0.05
	After drying, without phycocyanin	0.62^{e} ± 0.04
	Before drying, with phycocyanin	0.94^{a} ± 0.03
	After drying, with phycocyanin	0.81^{c} ± 0.06
L. plantarum	Before drying, without phycocyanin	0.91^{ab} ± 0.06
	After drying, without phycocyanin	0.67^{de} ± 0.05
	Before drying, with phycocyanin	0.98^{a} ± 0.04
	After drying, with phycocyanin	0.86^{bc} ± 0.05
L. casei	Before drying, without phycocyanin	0.89^{b} ± 0.04
	After drying, without phycocyanin	0.65^{e} ± 0.03
	Before drying, with phycocyanin	0.95^{a} ± 0.05
	After drying, with phycocyanin	0.80^{cd} ± 0.04

[†]Statistical analysis was performed for each strain separately. Values sharing the same letter within a given strain are not significantly different ($p > 0.05$). Comparisons were made vertically between conditions (Before drying vs. After drying, without or with phycocyanin) for the same strain

Discussion

The present study highlights the combined effects of storage temperature, drying, and phycocyanin supplementation on the viability, metabolic activity, surface properties, and biofilm integrity of *Lactobacillus* strains. In agreement with previous reports (Teneva and Denev, 2023 ; Kumar et al., 2023 ; De Bellis et al., 2021), storage temperature emerged as a major factor influencing probiotic stability, with freezing conditions providing the highest level of long-term preservation.

At room temperature, a rapid decline in probiotic viability was observed in the absence of protective agents, confirming the high sensitivity of *Lactobacillus* strains to oxidative and dehydration stresses during prolonged storage. Similar observations have been reported by Kharchenko et al. (2017), who demonstrated improved survival of *Bifidobacterium* spp. under frozen storage compared to conventional preservation methods. The stabilizing effect observed in the presence of 5% phycocyanin supports previous findings that natural bioactive compounds can mitigate stress-induced cell damage during storage (Teneva and Denev, 2023 ; De Bellis et al., 2021).

Beyond cell survival, maintaining metabolic functionality is crucial for probiotic efficacy. In the present study, lactic acid production was significantly better preserved in phycocyanin-supplemented samples, indicating improved retention of metabolic activity after drying. This observation is consistent with earlier work by Liu et al. (2011), who reported that phycocyanin can stimulate the growth and fermentation performance of lactic acid bacteria. Similarly, Shakirova et al. (2008) showed enhanced lactic acid production during fermentation in the presence of phycocyanin-rich *Arthrospira platensis*.

Vacuum drying significantly affected cell surface characteristics, as reflected by reduced adhesion to solvents. Changes in hydrophobicity and acid–base surface properties after dehydration have been previously reported (Mariam et al. [36]; Scherber et al. [37]) and are commonly associated with alterations in membrane lipids, surface proteins, and lipoteichoic acids. Such modifications may negatively impact the adhesion capacity of probiotics, which is a key determinant of intestinal colonization.

Importantly, phycocyanin supplementation partially preserved surface hydrophobicity after drying. This protective effect may be attributed to the antioxidant and membrane-stabilizing properties of phycocyanin, which have been described in several studies (Liu et al., 2011 ; Chakroun et al., 2022). By limiting oxidative damage and maintaining envelope integrity, phycocyanin may help preserve the physicochemical traits required for effective probiotic adhesion.

The analysis of biofilm-associated cells further supports the protective role of phycocyanin. Drying caused a significant reduction in biofilm viability, while supplementation mitigated this effect, particularly in *L. plantarum*. Biofilms are known to enhance bacterial stress tolerance and persistence (Rashtchi et al., 2024), and preservation of biofilm viability may therefore contribute to sustained probiotic functionality after processing.

Taken together, these results indicate that phycocyanin acts as a multifunctional protective agent, improving probiotic resistance to drying and storage stresses while maintaining key functional attributes. Nevertheless, as this study was conducted *in vitro* and limited to three strains, further investigations under simulated gastrointestinal conditions and *in vivo* models are required to confirm the relevance of these findings.

Conclusion

In conclusion, the present study demonstrates that storage conditions and drying processes markedly influence the viability and functional properties of *Lactobacillus* strains. Freezing was identified as the most effective method for long-term storage, while room-temperature storage resulted in rapid viability loss in the absence of protective compounds.

Supplementation with 5% phycocyanin significantly enhanced probiotic resilience by preserving cell viability, lactic acid production, surface hydrophobicity, and biofilm-associated metabolic activity during drying and storage. These effects highlight the potential of phycocyanin as a natural stabilizing agent for probiotic formulations. Further in *vivo* studies are required to validate its effectiveness under physiological conditions and to assess its impact on probiotic colonization and host interactions.

Conflicts of Interest

The authors declare no conflicts of interest.

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