Wiley
Journal of Food Processing and Preservation
Volume 2024, Article ID 7030605, 13 pages
https://doi.org/10.1155/jfpp/7030605

WILEY

Research Article

Effect of Coconut Powder as a Drying Carrier Medium on Long-Term Preservation of *Lactobacillus plantarum* and *Streptococcus thermophilus* for Development of Starter Cultures With Potential Application in Traditionally Fermented Foods

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Received 10 July 2024; Accepted 5 November 2024

Academic Editor: Nadica Maltar Strmečki

Fermentation is of great importance to the food industry as it extends the shelf life of food while imparting desirable nutritional and sensory properties. However, the unpredictable fermentation period, inconsistency of the final product quality, and contamination with spoilage microorganisms of spontaneously fermented food remain a challenge. The aim of this study was to explore the storage stability of *Lactobacillus plantarum* and *Streptococcus thermophilus* prepared by freeze-drying using coconut powder (CCN) (25%) as a carrier medium in comparison to skim milk powder (SMP). Freeze-dried lactic acid bacteria (LAB) were evaluated for cell viability, vitality, moisture content, water activity (a_w), acidification kinetics, cell morphology, and glass transition temperature (Tg). In general, LAB stored at 4°C showed better survival after 112 days than LAB stored at room temperature (RT). With cell viability, the reduction in *S. thermophilus* and *L. plantarum* counts was not significantly ($p < 0.05$) affected by the type of carrier media (CCN or SMP) after 112 days at 4°C. SMP supported better cell vitality (approx. 2.0) at 4°C than CCN after 112 days for *L. plantarum*. The moisture content and a_w were within acceptable levels throughout storage on all samples. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) depicted a normal cell structure at 4°C for *S. thermophilus* and *L. plantarum* in SMP and CCN, while negative changes were observed at RT. The Tg on all samples was above 75°C, and this suggested long-term stability of the LAB strains. Overall, our findings show that CCN shows similar protection of *L. plantarum* and *S. thermophilus* strains to that of SMP during storage at 4°C. Further work is needed to confirm the fermentation performance of CCN-preserved strains.

Keywords: coconut powder; freeze-drying; *Lactobacillus plantarum*; storage stability; *Streptococcus thermophilus*

Summary

- This article will give an insight into the development of starter culture.
- The process used will be applicable during the optimization of starter culture formulation, processing condition, and storage of freeze-dried cultures in consumer applications for traditional food fermentations.

1. Introduction

The food manufacturing industry uses starter cultures, mainly lactic acid bacteria (LAB), for the development of fermented food products such as yogurt, cheese, sourdough bread, and pickles. Fermenting food extends its shelf life and can also improve the nutritional and sensory properties of food. The starter cultures that are used for fermentation can be obtained in the iced state, ampules, and in a dehydrated form to prevent deterioration in quality during storage [1]. Generally, converting starter cultures and probiotics into freeze-dried bacteria powder has several benefits over frozen options due to easy handling and storage, convenience, easy movement through the supply chain, and more varied options for food inoculation. Dried bacteria cultures have the advantage of effectively prolonging the shelf life of products through extended protection of bioactive composites compared to liquid products [2, 3].

Skim milk powder (SMP) is one of the carrier media commonly used to preserve starter microbes during freeze-drying and subsequent low-temperature storage [4, 5]. Nonetheless, SMP is now facing competition from plant-based alternatives due to the fast-growing trend in consumers' daily diets for plant-based alternatives. In addition, milk contains caseins and β-lactoglobulin, with β-lactoglobulin being the major protein that causes allergic reactions [6]. Considering this, some studies have investigated the effect of nondairy media in preserving starter cultures. Sulabo et al. [7] showed the potential of mung bean powder as a preparation medium for drying starter cultures. These workers reported that the use of mung bean powder (5%) in combination with sucrose improved the fermentation potential (pH reduction and growth) of freeze-dried *Lactiplantibacillus plantarum* S20 when compared to nonfreeze-dried culture. The stability of *Lactobacillus fermentum* during freeze-drying was also investigated by Houngbédji et al. [8] using maize flour.

Coconut powder (CCN), on the other hand, is dairy-free and therefore a suitable alternative for the production of fermented food for those with dietary restrictions (milk allergy). Previous studies have shown that coconut milk can support the growth of LAB, including probiotic bacteria during fermentation [9, 10]. Also, coconut milk is rarely reported to cause allergic reactions in humans. In addition, other studies have confirmed that coconut milk can retain its nutrient content during storage for up to 3 weeks at low temperatures (4°C) [11]. Despite the reported potential of coconut milk to support microbial growth, there is paucity of information on the effectiveness of CCN in supporting the long-term storage of freeze-dried LAB starter cultures. Safeguarding the bacterium's integrity and survival during freeze-drying and subsequent storage is crucial for effective fermentation. To the best of our knowledge, there are no recent reports that determined the storage stability of freeze-dried LAB preserved with CCN as carrier media. The findings of this study could offer valuable insights into developing cost-effective and sustainable starter cultures for the food industry. Therefore, the study was aimed at developing a starter culture using a freeze-drying method and CCN as drying media and to determine the stability of LAB cultures at room temperature (RT) storage and 4°C.

2. Materials and Methods

2.1. Microorganisms. Lactobacillus plantarum and *Streptococcus thermophilus* were previously isolated, and cultures were selected from traditional fermented *Mahewu* and confirmed by 16sRNA sequencing. The strains were kept at −20°C in 20% glycerol (*v*/*v*) prepared with de Man, Rogosa, and Sharpe (MRS) broth. Before use, the LAB cultures were subcultured in MRS broth that was incubated at 37°C for 24 h and subjected to centrifugation and washing prior to freeze-drying.

2.2. Drying Media Preparation. CCN and SMP were purchased from the supermarket in Johannesburg, South Africa. SMP or CCN powder (25 g) was added into distilled water containing 0.85% NaCl (*w*/*v*). To decontaminate after mixing, the CCN mixture was heated to 121°C for 15 min, while skim milk was sterilized in a water bath to 90°C for 30 min [5]. Thereafter, each carrier medium was supplemented with sterile 5% sucrose (*w*/*v*) to a total volume of 200 mL at ±43°C. All the solutions were allowed to cool to a starting temperature for inoculation (±37°C) before the addition of the cell suspensions.

2.3. Inoculum Preparation and Freeze-Drying. Fresh LAB cells that had been grown on MRS agar were suspended in sterile saline water and adjusted to optical density, OD600 (10^9 CFU/mL). After that, 1 mL of the inoculum was aliquoted into the carrier medium, followed by freezing the mixture for 24 h at −20°C, and the cultures were frozen and dried singly. *S. thermophilus* was selected and used as a control LAB, while *L. plantarum* was selected because of its better performance results in other experiments carried out. Thereafter, the mixture was freeze-dried for 72 h using a freeze dryer (Telstar Lyoquest 55 y 85-2018) set at a drying temperature of −45°C. The resulting powders were then packed and vacuum-sealed in vacuum packs (linear low-density polyethylene [PE]) and kept at 4 ± 1°C and RT approx. 27 ± 1°C. Packed samples were sheltered from brightness by covering them with foil.

2.4. Determination of Cell Viability. The viability of the cells was tested before freeze-drying and during storage, as previously described by [12]. Briefly, 1.0 g of the sample was rehydrated in 9 mL sterile saline water (0.85% NaCl), followed by performing 10-fold serial dilutions in saline water. Subsequently, the spread plate method was performed by plating a 0.1 mL sample on MRS agar, and the plates were incubated at 37°C for 48 h. A colony count was performed, and the results were indicated as colony-forming units (CFUs) per gram. The following formula was utilized for calculating the survival percentage:

$$\%\text{of survival}(\log) = (\log \text{CFU/mL after freeze} - \text{drying}/\log \text{CFU/mL before freeze} - \text{drying}) \times 100$$

2.5. Determination of LAB Cell Vitality. To determine the cell vitality, the acidification power test that quantifies the glycolytic activity through the capability of cells to decrease the pH of a 0.1% glucose solution was used with slight modifications [13]. Briefly, the activated LAB cells suspended in MRS broth were harvested by centrifugation (from Centurion Scientific Limited, Monitoring and Control Laboratories) at $5000 \times g$ at 4°C for 10 min. The resulting pallets were washed three times with a quarter-strength ringer solution with the same centrifuge speed and time. Furthermore, to obtain a concentration of 10^7 cells/mL equivalent to OD600nm of 1.0 ± 0.2, the cells were transferred in quarter-strength ringer's solution at pH 6.4 for 10 min, and the pH was measured after 10 min of cell incubation at 25°C. The difference in pH between the first pH value of 6.4 and the pH_{20} value measured after 10 min of glucose addition was used to calculate the acidification powder test, and all values were tested in triplicate.

2.6. Determination of Moisture Content and Water Activity (a_w). To determine the moisture content, approx. 1 g of the sample was weighed and placed in an oven at 103°C for 24 h, and the constant weight obtained after oven drying was used to calculate moisture content in percentage [5]. a_w meter (MS-1 Set- aw, Novasina, Switzerland) was used to quantify a_w after stabilizing and calibration (with SAL-T 75%, 97%, 58%, and 11%) at ±26°C. The measurements were done in triplicate.

2.7. Determination of LAB Acidifying Activity. To assess the cells' ability to acidify 10% reconstituted skim milk, the acidifying activity was carried out according to a protocol described by [1], with some alterations. Skim milk (10%) was reconstituted and heated at 90°C for 1 h. Acidifying activity was determined for the freeze-dried cultures stored at 4°C and RT before freeze-drying and after 112 days of storage. The freeze-dried cultures were put on MRS broth, and then 1 mL was transferred to 10 mL of skim milk (w/v). Subsequently, the active acid generated was assessed with a pH at meters at 0, 6, and 24 h of incubation (30°C).

2.8. Scanning Electron Microscopy (SEM) Analysis. The microstructure of freeze-dried powders was examined by SEM (JSM 7500F VEGA3 TESCAN, Brno, The Czech Republic) following the method by [14]. Double-sided conducting carbon tape was placed on SEM aluminum stubs, then coated with palladium gold and observed using SEM. The cells were viewed at an acceleration voltage of 5 kV, with a working distance of ±15 mm, beam intensity of 8.00, and under resolution scan mode. The length of the cells was determined using ImageJ (Fiji) online software [15].

2.9. Transmission Electron Microscopy (TEM) Analysis. With TEM analysis, LAB cells from MRS broth in a pallet form were washed three times with 0.075 M phosphate buffer solution and fixed in 2.5% glutaraldehyde for 1 h [16]. Osmium tetroxide (1%) was used for 30 min to post-fix the cells. Pallets were dehydrated in graded sequences of ethanol (30%, 50%, 70%, 90%, and 3× with 100%) for 10 min each. Then, epoxy resin was mixed with the cells for 1 h. After that, fresh resin was added, and the samples were embedded in Eppendorf's tubes with a sample number for identification and oven-dried for 48 h at 55°C. Furthermore, the samples were cut into ultrathin sections and then stained with saturated aqueous uranyl acetate for 5 min, followed by rinsing in distilled water, drying, and transferring to lead citrate for 5 min, and finally examined under TEM (Jeol-2100F high-resolution transmission electron microscope [HRTEM], Tokyo, Japan).

2.10. Differential Scanning Calorimetry (DSC) Analysis. The DSC was used to determine the glass transition temperature (Tg) of freeze-dried LAB prepared with SMP and CCN [12]. Briefly, 1 g of the dry powders were hydrated in distilled water overnight, and then ±10 mg of the hydrated samples was placed in aluminum pans and hermetically sealed. The sealed samples were scanned from 0°C to 140°C at a heating rate of 10°C/min. Furthermore, for reference, an empty aluminum pan was utilized, and to calibrate the DSC equipment (DSC 3 STARe System, Mettler Toledo), a standard reference of indium was used.

2.11. Statistical Analysis. To see if there were any significant variations among the two-carrier media and LAB, analysis of variance (ANOVA) was utilized with IBM SPSS Statistics software. Multiple comparisons between groups were evaluated by Tukey's post hoc test. A p value < 0.05 indicated a significant difference. Data was presented as $mean \pm standard\ deviation$ ($n = 3$).

3. Results and Discussion

3.1. Changes in Cell Viability During Freeze-Drying of LAB Strains. The coconut powder's ability to protect LAB strains during freeze-drying compared to LAB prepared with SMP as a control was evaluated. In general, LAB preserved in both CCN and SMP maintained high counts (> 9 log CFU/g) after freeze-drying (Figure 1). In SMP, *S. thermophilus* maintained initial counts at the end of freeze-drying, while a significant ($p < 0.05$) reduction by 0.77 log CFU/g was observed on CCN powder. With *L. plantarum*, a significant reduction by 0.57 and 0.41 log CFU/g occurred after freeze-drying on SMP and CCN powder, respectively. The observed slight reduction in LAB counts after freeze-drying could be attributed to the occurrence of cell injury during ice crystal formation and the physical stress associated with the process [7, 17]. The findings of this study are similar to what was reported by Peralta et al. [18] on *Lacticaseibacillus paracasei 90*. These authors reported that the bacterium maintained high counts (> 9 log CFU/g) after freeze-drying and that cell viability during freeze-dying was not substantially affected by the drying medium.

The overall LAB survival rates after freeze-drying ranged between 97.12% and 99.82% on both carrier media (Figure 1). The highest survival (99.82%) occurred with *S. thermophilus* prepared with SMP, and the lowest was recorded with *L. plantarum* prepared with CCN. In general, the survival rates were strain-dependent, with *S. thermophilus* surviving better in SMP (51% protective effect) than in

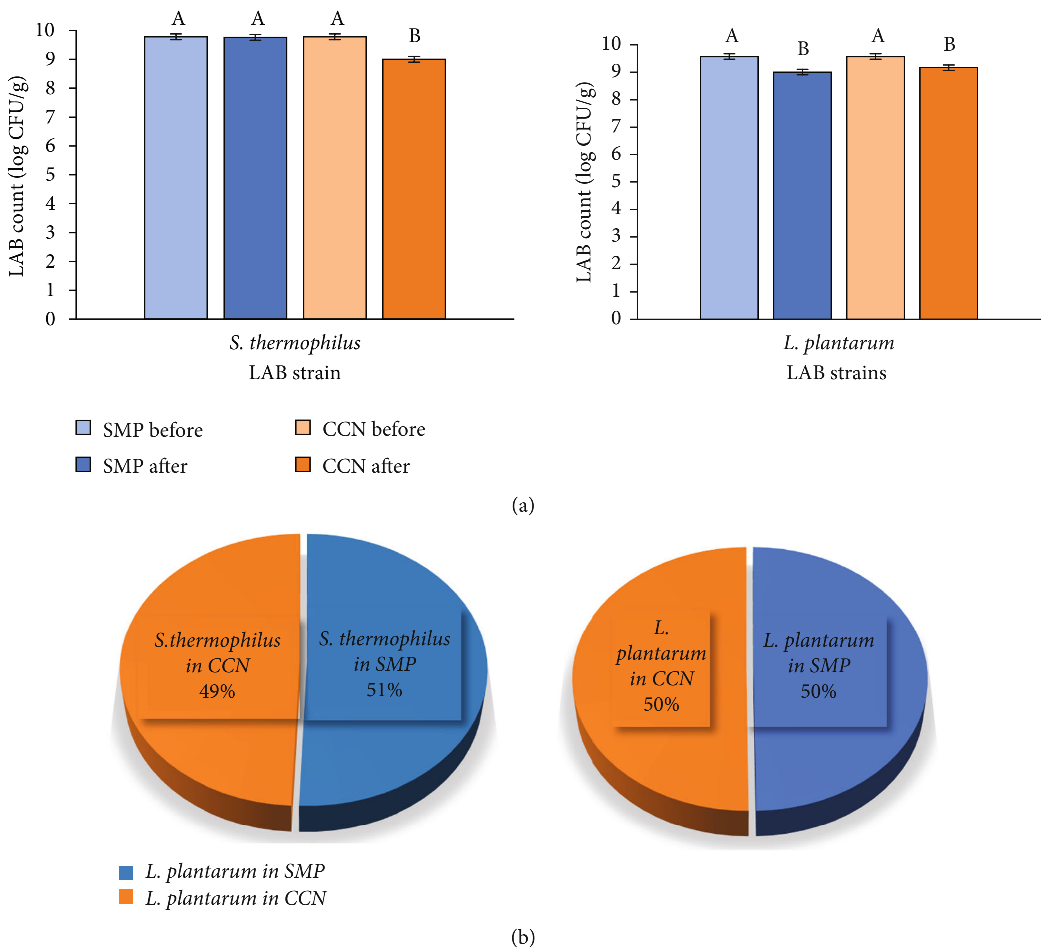

Figure 1: Effect of freeze-drying on (a) cell viability and (b) survival of LAB prepared with skim milk and coconut powder as drying medium. CCN, coconut powder; SMP, skim milk powder. The data represent means of three individual counts; the experiment was performed in triplicates. Different alphabets (^{a,b,c,d}) indicate a significant difference ($p < 0.05$).

CCN (49% protective effect). While there were no significant differences among the carrier media for *L. plantarum*, the protective effect for both SMP and CCN was 50%. Skim milk is generally thought to protect freeze-dried cells through the reduction of cell membrane damage by providing a protective layer of protein, thus stabilizing cell membrane components [19]. Previous research has shown the preservative potential of SMP on the protection of LAB strains during freeze-drying [12, 20]. Lu et al. [21] reported on improving *S. thermophilus* survival by adjusting the cryoprotective medium during freeze-drying. Their findings highlighted the positive influence skim milk and glycerol had on the survival of freeze-dried *S. thermophilus*, resulting in maximum cell survival of 93.58% after freeze-drying, which was lower compared to our results (99.82%). Although this survival rate was lower compared to our study, it still demonstrated the potential benefits of utilizing skim milk as a protective carrier medium for *S. thermophilus*.

3.2. Changes in Cell Viability During Storage of LAB Strains. Figure 2 shows the cell viability and survival of LAB strains preserved with CCN powder and SMP during storage at 4°C and RT for 112 days. As expected, the highest reduction in LAB counts occurred with LAB strains stored at RT when compared to 4°C, and this occurred with both preservation media. At RT, *S. thermophilus* counts on SMP and CCN powder decreased by 3.50 and 3.34 log CFU/g, respectively, after 112 days when compared to Day 0. At the end of storage, *S. thermophilus* counts on CCN powder were significantly lower than those on SMP. With *L. plantarum*, initial counts were maintained after 56 days at RT on CCN powder, followed by a significant reduction to 3.30 log CFU/g after 112 days. On SMP, a significant reduction in *L. plantarum* counts occurred after 56 days, followed by keeping statistically similar counts after 112 days. Except for what occurred after 56 days, there was no significant difference in *L. plantarum* counts on SMP and CCN powder.

At 4°C, *S. thermophilus* maintained initial counts after 84 days on both SMP and CCN powder. Thereafter, a similar but significant decrease (approx. 1.5 log CFU/g) in *S. thermophilus* counts occurred on both carrier media. With *L. plantarum*, there was no significant change in counts after 112 days on both SMP and CCN powder when compared to Day 0. However, a significant reduction in *L. plantarum* counts was observed after 28 days on CCN powder, followed by an increase in initial counts thereafter. The exact reason

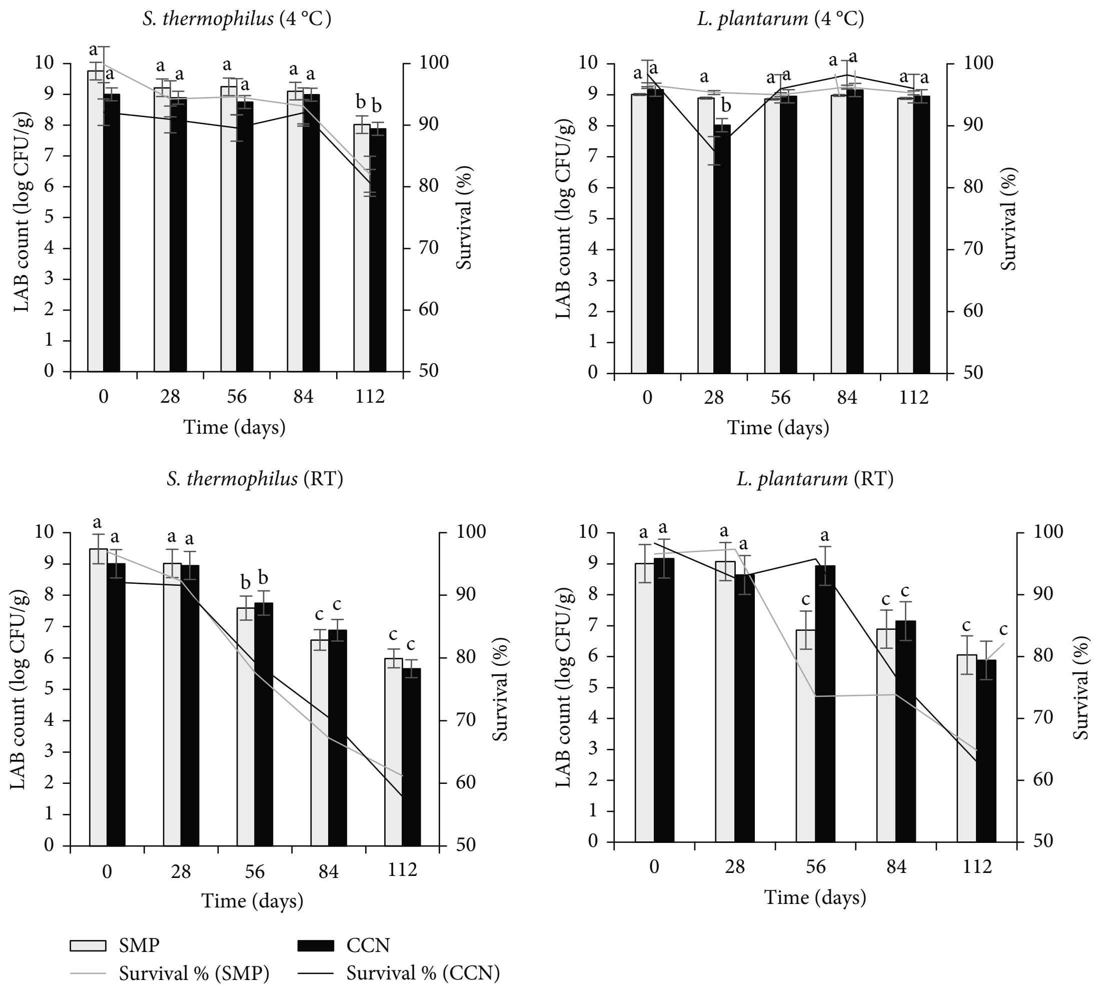

Figure 2: Cell viability count of lactic acid starter cultures preserved in skim milk powder (SMP) and coconut powder (CCN) by freeze-drying technique under two storage 4°C and room temperature (RT). *S. thermophilus*, *Streptococcus thermophilus*; *L. plantarum*, *Lactobacillus plantarum*. The data represent means of three individual counts; the experiment was performed in triplicates. Different alphabets ([a,b,c,d]) within a row indicate significant differences ($p < 0.05$).

for this is unknown but could possibly be due to the bacterium acclimatizing to the carrier medium.

The observed high reduction in counts at RT was thought to be due to the increases in the rate of biochemical reactions when compared to refrigeration storage [22]. Gaschler and Stockwell [23] reported that cells subjected to relatively high temperature show increased oxidation of fatty acid membrane lipids when compared to oxidation rates at low temperatures. This may lead to disruption of vital cellular processes since microbial chemical reactions are faster at higher temperatures [12]. The findings of this study agree with previous reports that reported decreases in cell viability when LAB was stored at RT. Gul et al. [12] investigated the storage stability of freeze-dried *L. brevis ED25* stored at 4°C and 25°C for up to 180 days using skim milk and sucrose as cryoprotectants. In their study, freeze-dried *L. brevis* cells survived better (0.14 log CFU/g reduction) at 4°C, while 1.17 log CFU/g reduction was reported with cells stored at 25°C [12].

Notably, the cell viability of *S. thermophilus* and *L. plantarum* on SMP and CCN powder was similar except for the difference that occurred with *S. thermophilus* after 112 days at RT. Previous reports have indicated that the type and even the concentration of cryoprotectants may affect the survival of LAB [24]. In this study, SMP was expected to provide more protection when compared to CCN due to the presence of milk proteins, lactose, salts, and vitamins. Milk proteins (caseins) provide a protective coating to cells against oxygen and other harm during storage [25]. In addition, it has been observed that lactose prevents injury to cells by preserving elements of the cell membrane, whereas whey protein disables frozen enzymes [26]. In contrast, the protein content of CCN powder is less (approx. 19.00%) than that of SMP (approx. 36.6%). The fact that CCP provided cell protection that was comparable to SMP suggests that the proteins in CCN as well as the presence of other compounds such as carbohydrates (16%–32%), fats (10%–15%), and ash (3%–9%) could have provided some protection to the freeze-dried LAB cells during storage [27].

The survival rate of freeze-dried *S. thermophilus* on SMP was 61.21% at RT after 112 days, while 57.91% survival was recorded on CCN powder after the same period (Figure 2).

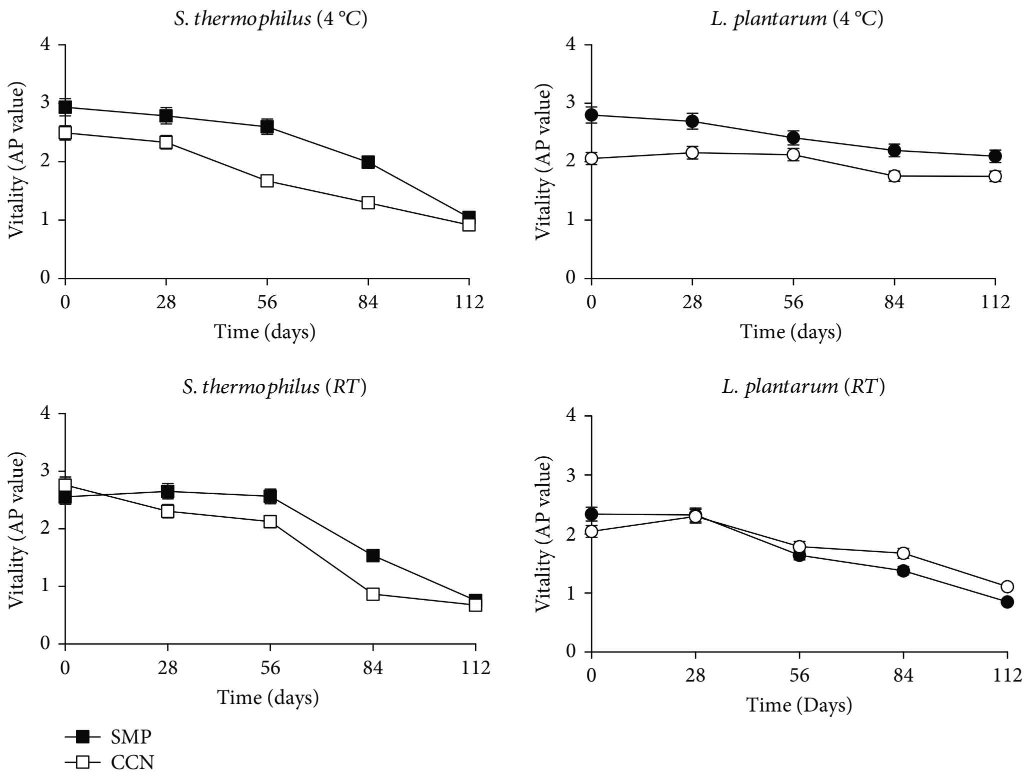

Figure 3: Cell vitality of lactic acid starter cultures preserved in coconut powder and skim milk powder by freeze-drying technique. Storage at 4°C and storage at room temperature. *Streptococcus thermophilus* and *Lactobacillus plantarum*. SMP, skim milk powder; CCN, coconut powder.

A survival of 64.91% and 63.05% occurred with *L. plantarum* on SMP and CCN powder, respectively, after 112 days at RT. *S. thermophilus* survival at 4°C was 82.92% on SMP, while 80.64% survival occurred on CCN powder. Overall, the highest survival rate occurred with *L. plantarum* on CCN powder at 4°C.

3.3. Changes in Cell Vitality During Storage of LAB Strains. In this study, an acidification power test was employed as a means to evaluate cell vitality, and the results are shown in Figure 3. In general, cell vitality decreased during storage with both LAB strains and preservation media. On SMP, cell vitality of *S. thermophilus* decreased by approx. 70% (2.55–0.76) at RT, while approx. 75% reduction (2.76–0.68) was observed in CCN powder after 112 days. With *L. plantarum* and on SMP, a 63% reduction in cell vitality (2.33–0.85) occurred at the end of storage at RT, while a 45% reduction (2.04–1.11) was recorded on CCN powder. At 4°C, the cell vitality of *S. thermophilus* decreased by approx. 64% on SMP, while a 63% (2.49–0.92) decrease occurred on CCN after 112 days. *L. plantarum* cells on SMP showed a 25% decrease at 4°C after 112 days and a 24% (2.05–1.95) decrease on CCN powder after the same period. Overall, the current findings indicate that the extent of cell vitality loss was higher at RT on cells preserved on CCN powder than SMP. In contrast, cell vitality loss was similar on CCN powder and SMP at 4°C. Also, the cell vitality of *L. plantarum* was least affected during storage, on both SMP and CCN powder. However, it must be noted that with *S. thermophilus*, low vitality does not always represent poor metabolic activity because the bacterium is an obligate heterofermentative bacteria. This means it produces less acid, which is the basis for the method here, than homofermentative LAB [13]. According to Gabriel et al. [28], a viable culture with strong fermentation capacity is demonstrated by a vitality value of 2.0–2.7, whereas AP values less than 2 signify a decreased activity in metabolism. In this study, the cell vitality of all samples after 112 days was less than 2.0. This means that storage of up to 84 days at 4°C should be considered to avoid negatively affecting cell vitality.

3.4. Changes in Moisture Content and a_w During Storage of LAB Strains. During storage, moisture content plays a significant role in influencing cell viability and maintaining product solidity [12, 29]. In general, the moisture content of all the samples in this study ranged from 2.81% to 4.72% (Table 1). The moisture content of *S. thermophilus* on both SMP and CCN powder decreased by approx. 0.88% at RT, while a similar reduction in *L. plantarum* counts was observed on both carrier media. At 4°C, *S. thermophilus* showed a significant moisture content increase (0.87%) on SMP but a 0.87% decrease in counts in CCN powder. For *L. plantarum* on SMP and CCN powder, a similar increase in moisture content of 0.91% was observed after 112 days. Overall, the moisture content changes that occurred during storage were approx. 1% for all samples, and this was attributed to the type of packaging used. According to Wu, Misra, and Mohanty [30], PE vacuum

Table 1: Moisture content (percentage) and water activity of freeze-dried LAB strains preserved in skim milk and coconut powder throughout storage at room temperature and 4 ± 1°C for 112 days.

Analysis	Storage	LAB strain	Storage period (days) 1	28	56	84	112
Moisture content	Room temperature	*S. thermophilus*, SMP	3.69 ± 0.49^{b}	3.40 ± 0.49^{a}	3.09 ± 0.99^{a}	3.47 ± 0.74^{a}	2.81 ± 0.76^{a}
		S. thermophilus, CCN	4.04 ± 1.02^{b}	4.08 ± 1.08^{b}	3.92 ± 0.48^{b}	3.21 ± 0.50^{a}	3.18 ± 0.14^{a}
		L. plantarum, SMP	3.79 ± 1.08^{b}	3.15 ± 1.08^{a}	3.20 ± 1.40^{a}	3.18 ± 1.76^{a}	2.84 ± 1.00^{a}
		L. plantarum, CCN	4.20 ± 0.65^{b}	4.17 ± 0.15^{b}	3.79 ± 0.57^{b}	3.21 ± 0.45^{a}	2.92 ± 0.75^{a}
	4 ± 1°C	*S. thermophilus*, SMP	3.69 ± 0.49^{b}	3.41 ± 0.49^{a}	3.39 ± 0.68^{a}	3.27 ± 1.92^{a}	4.44 ± 1.10^{c}
		S. thermophilus, CCN	4.68 ± 0.63^{c}	4.68 ± 1.08^{c}	4.72 ± 0.19^{c}	3.53 ± 1.11^{b}	3.81 ± 1.65^{b}
		L. plantarum, SMP	3.15 ± 0.04^{a}	3.18 ± 1.08^{a}	3.19 ± 1.04^{a}	3.28 ± 0.56^{a}	4.06 ± 2.40^{b}
		L. plantarum, CCN	3.15 ± 0.04^{a}	3.15 ± 1.08^{a}	3.29 ± 1.04^{a}	3.28 ± 0.56^{a}	4.06 ± 2.40^{b}
Water activity	Room temperature	*S. thermophilus*, SMP	0.27 ± 0.34^{a}	0.29 ± 0.43^{a}	0.27 ± 0.53^{a}	0.28 ± 0.51^{a}	0.23 ± 0.02^{a}
		S. thermophilus, CCN	0.25 ± 0.01^{b}	0.33 ± 0.02^{b}	0.37 ± 0.03^{d}	0.36 ± 0.97^{d}	0.24 ± 0.03^{b}
		L. plantarum, SMP	0.28 ± 0.06^{a}	0.27 ± 0.04^{a}	0.31 ± 0.02^{a}	0.31 ± 0.07^{a}	0.32 ± 0.05^{a}
		L. plantarum, CCN	0.18 ± 0.05^{c}	0.23 ± 0.03^{c}	0.25 ± 0.04^{b}	0.27 ± 0.05^{b}	0.21 ± 0.03^{c}
	4 ± 1°C	*L. plantarum*, SMP	0.28 ± 0.03^{a}	0.29 ± 0.10^{a}	0.28 ± 0.41^{a}	0.27 ± 0.11^{a}	0.27 ± 0.03^{a}
		L. plantarum, CCN	0.18 ± 0.02^{c}	0.23 ± 0.04^{c}	0.22 ± 0.04^{c}	0.23 ± 0.06^{c}	0.26 ± 0.03^{b}
		S. thermophilus, SMP	0.25 ± 0.01^{a}	0.25 ± 0.03^{a}	0.25 ± 0.22^{a}	0.26 ± 0.21^{a}	0.25 ± 0.13^{a}
		S. thermophilus, CCN	0.25 ± 0.11^{b}	0.26 ± 0.06^{b}	0.27 ± 0.08^{b}	0.27 ± 0.12^{b}	0.30 ± 0.71^{b}

Note: Different alphabets (^{a,b,c,d,}) within a row indicate significant differences ($p < 0.05$), ± standard deviation ($n = 3$).
Abbreviations: CCN, coconut powder; SMP, skim milk powder.

pouches are highly impermeable to moisture and thus have a lower water vapor transmission rate. The slight increases in moisture content that occurred at 4°C could be due to a slight ingress of moisture through the packaging since the relative humidity is high at refrigeration. Yang et al. [31] reported that the high survival rate of dried *Tetragenococcus halophilus* bacterial powder stored in a refrigerator (4°C) for 8 weeks was attributed to maintaining a moisture content within the range of 2.8%–5.6%. Notably, the moisture content of all samples in this was within this range throughout storage, which suggests that they should be stable.

The a_w of the freeze-dried cultures monitored during storage is shown in Table 1. In general, all the freeze-dried starter cultures showed a relatively low a_w that ranged between 0.23 and 0.32. At RT, there was no significant difference in a_w during storage on *S. thermophilus* preserved on both SMP and CCN powder. Similarly, *L. plantarum* on SMP and CCN powder did not show a significant difference in a_w during storage. With samples stored at 4°C, there were no significant differences in a_w during storage on all samples, with the highest a_w (0.30) observed after 112 days with *S. thermophilus* preserved in CCN powder. In general, the highest a_w of all samples in this study was 0.32. According to Muhammad et al. [32], an optimum a_w of below 0.3 is required to reduce biochemical processes and extend shelf life. Based on this, the sample of this study is expected to have an extended shelf life. A much lower a_w (0.12–0.17) than the current study was reported for *L. plantarum* stored at 4°C for 60 days [1]. The difference in results could be attributed to the differences in the packaging used (closed safe–lock Eppendorf tubes) and carrier media (sweet whey powder and sweet whey permeate). In another study, Üçok and Sert [5] reported a higher a_w (0.55) than that of the current study for *L. plantarum* preserved in whey powder that was stored at 4°C for 150 days. The high a_w in their study negatively influenced the survival rate of *L. plantarum* with a noticeable decline in counts after 30 days.

3.5. Determination of Acidifying Activity of the LAB Strains at the End of Storage. The acidifying activity of LAB cultures at the end of storage (4°C and RT for up to 112 days) was determined by accessing the dried culture's ability to acidify 10% skim milk, compared with the fresh (control) LAB indicated in Table 2. Previous reports have indicated that bacteria that can reduce the pH of milk at 30°C to less than 5.3 after 6 h can support a good fermentation. In this study, *S. thermophilus* preserved on both SMP and CCN powder and stored at 4°C managed to reduce the pH to less than 5.3 in 6 h, while that was not the case with *S. thermophilus* stored at RT. Similar findings were observed with *L. plantarum.* As expected, fresh LAB strains showed a rapid reduction in pH than LAB strains that had been stored for 112 days.

With fresh LAB, a rapid reduction in pH (6.17–2.98) occurred with *L. plantarum* on SMP, while a 6.34–3.59 pH reduction occurred on CCN after 24 h. The acidification activity of fresh *S. thermophilus* was reduced to 3.47 in SMP and to 4.39 in CCN. In comparison to the old strains, *S. thermophilus* in SMP reduced the pH to 5.97 and 5.85 within 6 h for SMP and CCN at RT, respectively. A more rapid reduction from 6.28 to 4.66 for SMP and 6.16 to 4.98 was observed for *S. thermophilus* (4°C) within 6 h. The old strains of *L. plantarum* showed a reduction in acidification within 6 h with a pH change from 6.08 to 5.69 and from

Table 2: Acidification activity (pH) of LAB prepared with skim milk as carrier medium stored in different temperatures.

LAB strains	Control (before storage)			End of storage (RT) Storage time (h)			End of storage (4°C)		
	0	6	24	0	6	24	0	6	24
S. thermophilus SMP	6.17 ± 0.02[a]	5.06 ± 0.04[b]	3.47 ± 0.19[d]	6.08 ± 0.02[a]	5.97 ± 0.08[a]	5.10 ± 0.35[b]	6.28 ± 0.04[a]	4.66 ± 0.58[c]	4.10 ± 0.27[d]
S. thermophilus CCN	6.24 ± 0.39[a]	4.74 ± 0.18[c]	4.39 ± 0.06[c]	6.18 ± 0.34[a]	5.85 ± 0.23[b]	5.87 ± 0.27[b]	6.16 ± 0.10[a]	4.98 ± 0.06[b]	4.11 ± 0.10[d]
L. plantarum SMP	6.17 ± 0.02[a]	4.42 ± 0.20[c]	2.98 ± 0.27[d]	6.08 ± 0.02[a]	5.69 ± 0.17[b]	4.88 ± 0.13[b]	6.28 ± 0.04[a]	4.89 ± 0.11[b]	3.78 ± 0.20[d]
L. plantarum CCN	6.24 ± 0.39[a]	4.48 ± 0.10[c]	3.59 ± 0.10[d]	6.18 ± 0.34[a]	5.99 ± 0.10[a]	5.02 ± 0.02[b]	6.16 ± 0.10[a]	5.27 ± 0.13[b]	3.72 ± 0.06[d]

Note: Different alphabets ([a,b,c,d]) within a row indicate significant differences ($p < 0.05$), ± standard deviation ($n = 3$).
Abbreviations: CCN, coconut powder; SMP, skim milk powder.

6.18 to 5.99 for SMP and CCN at RT storage, respectively. While for the 4°C storage, the pH reduced to 4.89 for SMP and to 5.27 for CCN within 6 h. Overall, the findings of this study suggest that low-temperature storage can preserve the activity of the starter cultures, leading to better acidification kinetics. Other studies have reported similar results with stored freeze-dried *L. brevis* subjected to 17.28% skim milk [12].

3.6. Observation of Change in Cell Morphology of LAB Using SEM. The cell morphology of the LAB strains stored for up to 112 days was compared to the control (Day 0), as shown in Figure 4. *L. plantarum* cells preserved in SMP showed long rods with a smooth surface and an average length of 4.39 μm at Day 0. The cells maintained a rod shape and smoothness throughout the storage at 4°C, which possibly explains the high cell viability observed with *L. plantarum* (Figure 2). However, the average cell length for *L. plantarum* in SMP was reduced from 4.39 to 3.01 μm at 4°C and to 2.85 μm at RT after 112 days of storage. The reduction in cell length could be indicative of some stress response during storage. Interestingly, *L. plantarum* cells preserved with CCN powder showed a similar cell length reduction from approx. 4.46 to 3.80 μm after 112 days under storage conditions. The elongated cellular morphology in the dried lactobacilli strains could reflect the rapid division of cells [3]. This action may serve as a protective mechanism that explains the maintained cell morphology of *L. plantarum*.

S. thermophilus cells showed no change in cell morphology after 112 days of storage in SMP at 4°C, with average cell length maintained at approx. 1.88 μm, similar to what occurred with CCN, there was no change in cell length for *S. thermophilus*, and the cell length was maintained at 1.95 μm. *S. thermophilus* cell in SMP at RT was reduced from 1.88 to 1.42 μm after 112 days. With RT storage, *S. thermophilus* prepared with CCN showed a significant change in cell morphology and cell shrinkage with a cell length reduction from 1.95 to 1.4 μm. In addition, the cells after 112 days of storage were not smooth compared to Day 0 cells. This rough surface detail is linked to wrinkling of the cell wall due to shrinkage.

3.7. Observation of Cell Structure Using TEM. To further investigate the influence of storage temperature on the cell structure, the internal structure of the LAB cells was analyzed using TEM. The fresh *L. plantarum* cells preserved in SMP at 4°C appeared to have an intact cell (Arrow a) and cell membrane along with a well-distributed cytoplasm (white material, Arrow c), as shown in Figure 5. Also, with fresh cells of CCN powder, all the LAB strains stored at 4°C showed a uniform cytoplasm and an intact cell wall. This confirmed the normal cellular morphology of the cells at the beginning of the storage. After 112 days at 4°C, *L. plantarum* cells in SMP had a clear nuclear material (Arrow b) and cytoplasm content (Arrow c), which meant that their internal cell contents were intact. Cell split was noticed with *L. plantarum* cells in CCN powder at 4°C after 112 days of storage (Arrow e), while the cytoplasm material remained well distributed. At RT storage, *L. plantarum* cells in CCN powder exhibited moderate damage to the cell wall after 112 days (Arrow d). However, their cell membrane remained intact. In contrast, the internal structure of *L. plantarum* cells in SMP stored at RT showed a slightly lysed cell wall and cell membrane (Arrow d). This indicates that with time, the integrity of the *L. plantarum* cells was negatively affected in this carrier medium. The presence of an intact cell membrane plays a crucial role in preventing the spillage of the cytoplasmic content, and such cells are able to survive throughout storage [33].

As expected, *S. thermophilus* cells at Day 0 showed a cocci shape with intact cell walls and cell membranes (Arrow a, *S.t*). Notably, *S. thermophilus* cells preserved in SMP maintained the coccus shape after 112 days at 4°C and did not show any intracellular damage. The nuclear material (Arrow b, *S.t* 4°C) and cytoplasm of *S. thermophilus* at 4°C prepared with CCN powder appeared as globules of variable size with intact cell walls (Arrow a). This was, however, not the case with *S. thermophilus* cells at RT in SMP as they showed some cell wall damage with a frayed appearance (Arrow d, *S.t* RT), indicating a probable peptidoglycan modification. Furthermore, *S. thermophilus* cells stored at RT in CCN powder exhibited an altered cell wall and damaged cell membrane with fibre-like protrusions and leakage of

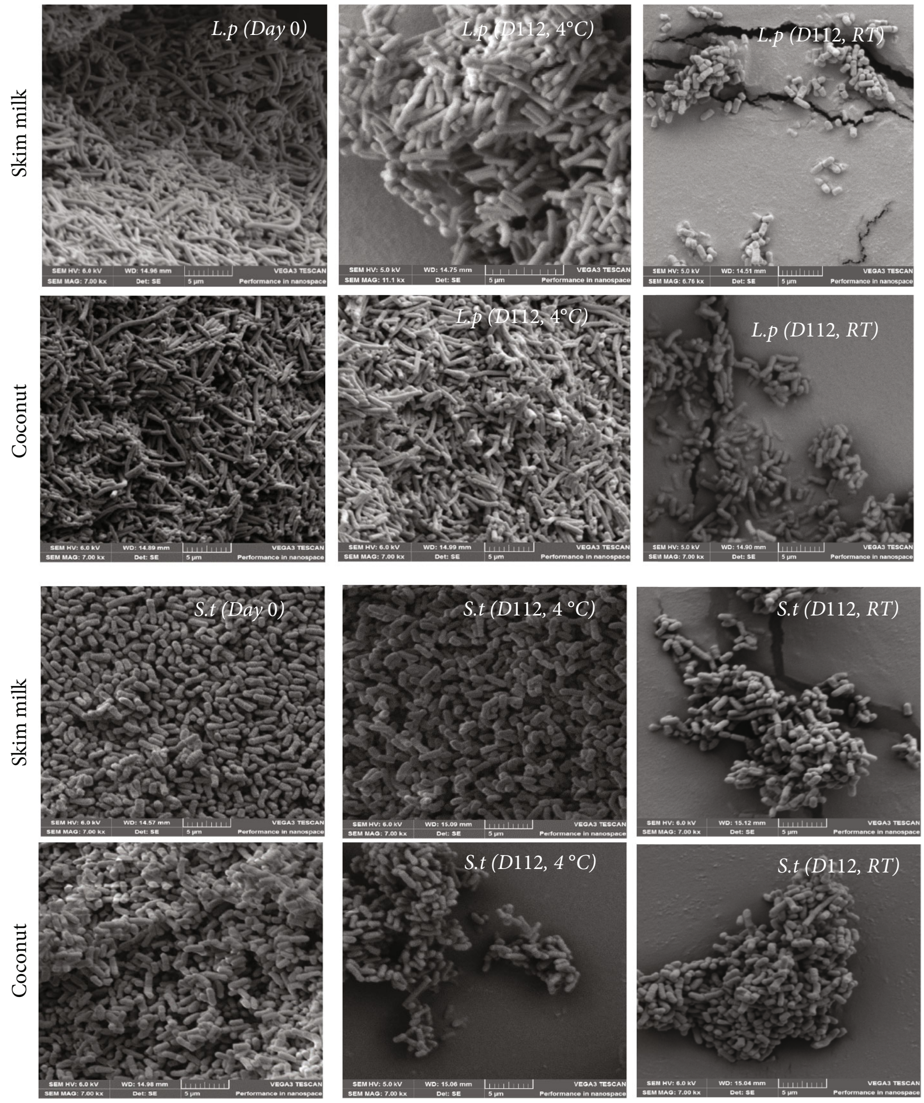

Figure 4: Scanning electron microscopy images showing changes in cell morphology of LAB before storage (Day 0) and 112 days of storage (D112). L.p, *Lactobacillus plantarum*; S.t, *Streptococcus thermophilus*; RT, room temperature storage and storage at 4°C.

intracellular contents (Arrow f). The observed bubble-like structures on the cell surface of *S. thermophilus* were thought to be due to cell shrinkage and rupture [32].

In a study by Yang et al. [34], these three carriers (10% skimmed, 8% sucrose, and 0.1% L-sodium glutamate) were demonstrated to maintain the cell integrity of freeze-dried *L. plantarum* LIP-1 strains stored at RT storage for 7 weeks. These workers attributed the improved preservation to low ROS content, which slowed the oxidation rate of fatty acids in the cell membrane, thus preserving the integrity and fluidity of cells. Similarly, *L. plantarum* in the current study also showed an intact cell wall at RT (Figure 5, *L.p* RT). Based on the literature, the influence of the fatty acid profile on maintaining cell membrane fluidity and high integrity is one of the mechanisms required by cells to survive during drying and storage condition stresses [35]. In this study, the CCN used contained 13.4% total fat which explains the observed cell integrity.

The deformed cell wall and cell membrane noticed with *L. plantarum* in CCN powder could result in cell death because the cell wall of LAB is generally made up of several structural components [36]. Briefly, these components include peptidoglycan, various polymers like polysaccharides and lipoteichoic acids, and exopolysaccharides on the outer surface [22]. These components play an important role in protecting bacterial shape and integrity during storage and serve as a protective barrier between the bacterium and its environment [36].

Based on the cell morphology observed under SEM and TEM, the best storage condition for LAB was 4°C and for up to 112 days in SMP. However, it must be noted that CCN powder showed better protection of cell morphology, but at 4°C only. In general, there was a bacterial variation observed in the cell morphology. The intact cell wall and cell membrane of *S. thermophilus* were maintained under both storage conditions when SMP was used. The SEM results

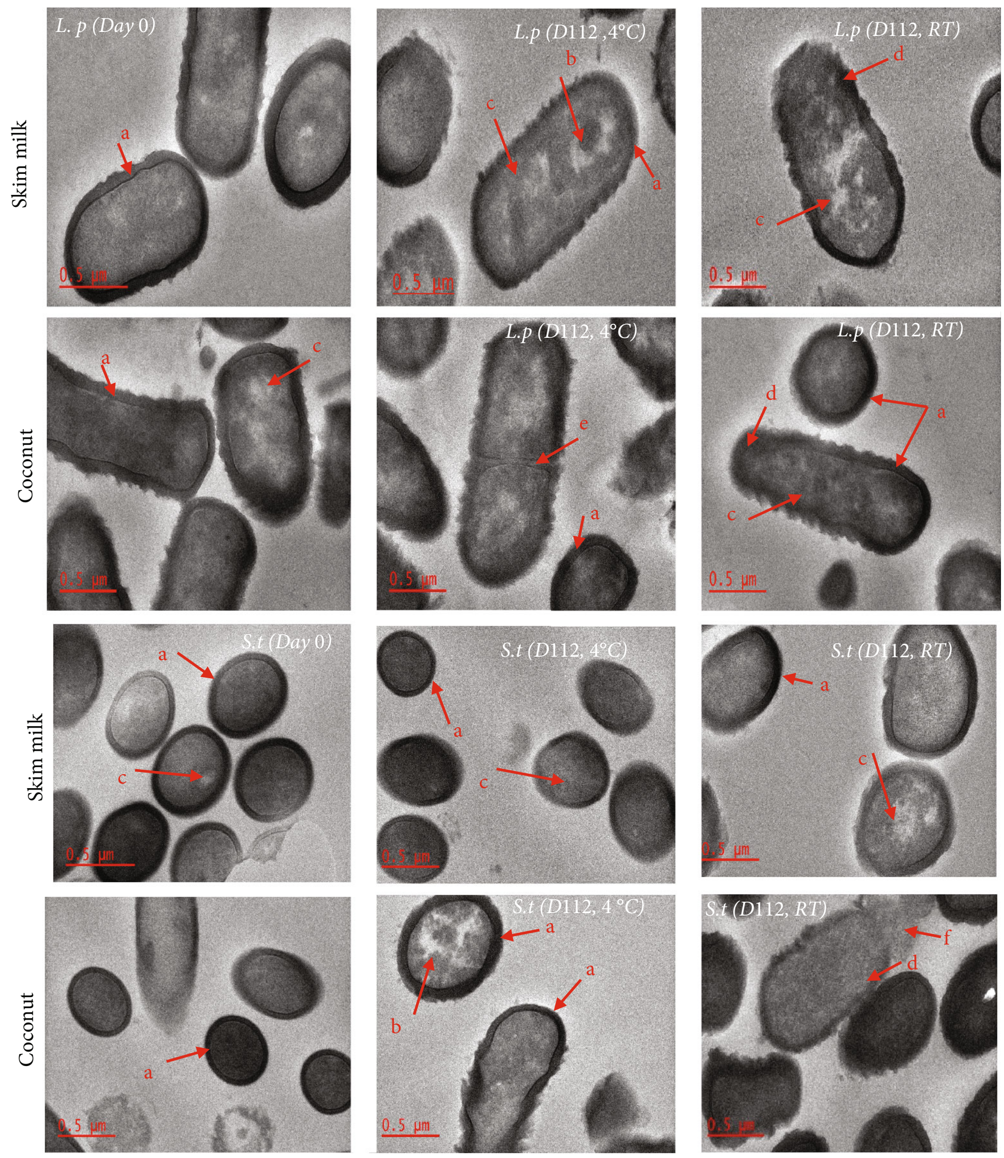

Figure 5: Transmission electron microscopy images showing changes in cell morphology of LAB before storage (Day 0) and 112 days of storage (D112). L.p, *Lactobacillus plantarum*; S.t, *Streptococcus thermophilus*; RT, room temperature storage and storage at 4°C. Arrow a, intact cell wall and cell membrane; Arrow b, nuclear material; Arrow c, cytoplasm content; Arrow d, lysed cells; Arrow e, splitting cells; Arrow f, leakage of intracellular contents.

correlate with the viability and vitality of *L. plantarum*, where the viability was maintained throughout the storage at 4°C for both SMP and CCN powder. The significant change in cell length and cell surface for SEM results explains the significant decline in cell viability and vitality for *L. plantarum* and *S. thermophilus* under RT storage.

3.8. Effect of Storage on the Glass Transition Behavior of Freeze-Dried LAB. Tg is regarded as an essential metric for assessing the stability of freeze-dried bacterial powder [12, 37]. It is defined as the temperature at which an amorphous substance, such as a dry culture, changes from a glassy, rigid condition to a rubbery or viscous state [38]. Therefore, it represents a state in which the dried culture becomes more mobile to potentially undertake chemical and biological interactions. In this study, only LAB cells that were stored at 4°C were selected for Tg because they resulted in better viability and improved vitality and acidification kinetics when compared to RT storage. Overall, the Tg decreased by an average of 7.10°C with storage, and a sharp endothermic peak was observed at above 75°C, corresponding to the Tg for all the LAB (Table 3). The Tg of LAB prepared with SMP was reduced by 9.86°C for *S. thermophilus* and with CCN powder decreased by 8.61°C for *S. thermophilus*. The Tg of *L. plantarum* prepared with SMP was reduced by 5.99°C, while for the same strain in CCN, the Tg decreased by 4.11°C. A much lower Tg was reported previously, with a peak at 64.56°C for freeze-dried *L. brevis ED25* powder [12]. Though their Tg was lower compared to the current work, it was quite above the storage temperature which

Table 3: Glass transition temperatures of the dried powder prepared by freeze-drying.

Carrier medium	Strain	Before storage Tg (°C) Onset	After 112 days of storage at 4°C Tg (°C) Onset
Skim milk powder	*S. thermophilus*	88.95 ± 1.00	79.27 ± 0.07
	L. plantarum	90.77 ± 0.96	84.78 ± 0.11
Coconut powder	*S. thermophilus*	91.80 ± 0.63	83.19 ± 0.09
	L. plantarum	93.91 ± 0.01	89.80 ± 0.11

indicates the long-term stability of the strain. This was attributed to the presence of lactose used in their study.

The study highlights the correlation between Tg and the viability of freeze-dried LAB. A high Tg is linked to elevated cell viability and survival rates, suggesting that maintaining a glassy state in the course of dehydration and storage preserves the biological components' integrity [31]. The observed correlation between Tg, cell viability, and vitality indicates a decrease in viability and potential impairment of cell survival with prolonged storage. Despite a decreasing trend, the relatively high Tg at 4°C explains the sustained cell viability. When compared to other studies, the Tg observed in this study was high, and this was probably related to the presence of sucrose in the formulation. Sucrose is known to serve as a dehydrating agent, lowering the amount of intracellular water and forming a glassy matrix as a protectant for membranes [38]. The study recommends keeping the Tg between 75°C and 95°C for freeze-dried LAB, emphasizing that a Tg above 75°C helps maintain cell wall integrity and viability, ensuring protective capabilities during storage.

4. Conclusion

Overall, storing the LAB strains at 4°C resulted in better storage stability compared to RT. Preservation of *S. thermophilus* and *L. plantarum* on CCN powder was found to be comparable to SMP with respect to cell viability, cell vitality, and acidifying activity. However, for effective cell vitality, storage of up to 84 days is recommended. The SEM and TEM results indicated normal cellular morphology of the cells after freeze-drying and subsequent storage at 4°C. In general, all LAB cultures maintained a high Tg in both carrier media at the end of storage, which was indicative of long-term stability. The findings of this study indicate that storage at 4°C provides better retention of starter culture quality and preservation of LAB strains with CCN powder, providing similar cell protection to that of SMP during storage. Further investigations must be carried out to improve the formulations for better cell stability at RT. In addition, the fermentation potential of LAB preserved with CCN powder must be determined in food.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Nontobeko Xolisiwe Zulu: conceptualization, methodology, investigation, sample analysis, writing—the initial draft, result analysis and interpretation. Angela Parry-Hanson Kunadu: conceptualization, writing—review and editing. Eugenie Kayitesi: writing—review and editing. Bhekisisa Dlamini: conceptualization, methodology, writing—review and editing, supervision.

Funding

This study was funded by the National Research Foundation, South Africa (Grant No. MND200619533859).

Acknowledgments

The authors would like to acknowledge the National Research Foundation, South Africa, for funding the current study. The University of Pretoria is also acknowledged for assisting with sample preparation for the transmission electron microscope. The authors would also like to thank the Department of Chemical Science at the University of Johannesburg for assisting with sample viewing for the scanning electron microscope and transmission electron microscope.

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