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A Comparative Study of Skimmed Milk and Cassava Flour on the Viability of Freeze-Dried Lactic Acid Bacteria as Starter Cultures for yogurt Fermentation

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Abstract: Concentration and preservation of lactic acid bacteria (LAB) starter cultures for food production guarantee the long-term delivery of stable cultures in terms of viability and functional activity. One method that has commonly been used to prepare dried starter cultures for food applications is freeze-drying. During freeze-drying, the bacterial cells are exposed to stresses such as freezing, drying, and long-term exposure to low-water activity. Additionally, another added stress is introduced during the rehydration process. The purpose of this study was to evaluate the survival rates and fermentation performance of three (3) freeze-dried lactic acid bacterial cultures previously isolated from Ghanaian traditional fermented milk. LAB cultures, i.e., *Lactobacillus delbrueckii*, *Lactococcus lactis* and *Leuconostoc mesenteroides* were frozen in the chamber of a Telstar (Lyoquest) laboratory freeze dryer for 10 hrs at -55 °C (as single and combined cultures) using skimmed milk and cassava flour as cryoprotectants held in plastic or glass cryovial. For viability during storage, freeze-dried LAB cultures were stored in a refrigerator (4 °C) and at room temperature (25 °C) for 4-weeks. The survival of freeze-dried cultures was determined by growth kinetics at 600nm (OD_{600}). The performance of freeze-dried LAB cultures after 4-weeks storage was determined by their growth and acidification of milk during yogurt fermentation and consumer sensory evaluation of fermented milk using a 9-point hedonic scale. The survival rates for LAB ranged between 60.11% and 70.91% following freeze-drying. For single cultures, the highest survival was recorded for *Lactobacillus delbrueckii* (L12), whereas for combined cultures, the highest survival was observed for *Lactococcus lactis* (L3) combined with *Lactobacillus delbrueckii* (L12). During the fermentation process all the freeze-dried LAB cultures were able to acidify yogurt to a pH below 4, while yogurt produce from the spontaneous fermentation was characterized by low acidification. Yogurt fermented with freeze-dried lactic acid bacteria cultures, either single or combined strains, showed improved acceptability as compared to the spontaneously fermented yogurt. The consumer acceptability results showed that yogurts produced with combined starter culture of *Lactoccus lactis* and *Lactobacillus delbrueckii* or single culture of *Lactococcus lactis* were the most preferred products with *Lactococcus lactis* and *Lactobacillus delbrueckii* possessing high survival rates and high consumer acceptability in yogurt production. These findings are crucial and can be adopted for large-scale production and commercialization of yogurt production, however, in-depth investigation on the effects of freeze-drying and long-term storage on survival and performance of selected LAB cultures are needed.

Keywords: lactic acid; freeze-drying; yogurt starter culture; fermentation

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Introduction

Fermentation plays a significant role in the traditional processing of food in many parts of the world. In many developing countries, traditional fermentation also serves as the method for improving the shelf life of many staple foods, whilst improving their digestibility, nutritional qualities, organoleptic properties and degradation of toxins and antinutritive factors (**Zang et al., 2020, Maicas 2020, Adebo 2020, Voidarou et al., 2020**). However, in Ghana and many parts of Africa, traditional fermentation processes are natural, that is, without the use of starter cultures (back slopping), which have implications for the safety and quality of the products (**Wirawati et al., 2019, Kim et al., 2018, Özel et al., 2020, Venema & Surono, 2020**).

In Ghana, naturally fermented milk is commonly produced and consumed by people living in cattle-rearing communities (**Agyei et al., 2020, Motey et al., 2021, Sessou et al., 2019, Ayivi et al., 2020**). The production of traditional yogurt-like milk products in Ghana does not rely on the use of commercial starter cultures. Therefore, stocks of previous ferments, fermentation containers, and environmental microorganisms contaminating the raw material often initiate fermentation in new batches (**Owusu-Kwarteng et al., 2020**). During the process, raw or pasteurized milk is kept in calabashes or plastic containers, covered with a lid, and allowed to spontaneously ferment at ambient temperature (28-35°C) for about 18-24 h. This natural fermentation results in the formation of curdled milk, yielding a slightly sour yogurt-like product with pH of less than 4 and varying consistency (**Agyei et al., 2020, Motey et al., 2021, Sessou et al., 2019, Ayivi et al., 2020, Owusu-Kwarteng et al., 2020**).

Generally, the dependence on such an undefined and diverse microbial consortium during fermentation results in products with inconsistent quality and stability (**Abi Khalil et al., 2022, Galli et al., 2022**). In a framework to develop starter cultures for controlled fermentation and production of fermented yogurt-like milk with greater consistency in quality and safety, it is required that LAB cultures for commercial and consistent fermentation processes are adequately propagated and made available as concentrates, either in a frozen or freeze-dried form (**Vinderola et al., 2019, Chen & Hang 2019**). Concentration and preservation of LAB starter cultures for food production rely on technologies, which guarantee the long-term delivery of stable cultures in terms of viability and functional activity (**Brizuela et al., 2021, Terpou et al., 2019, Fonseca et al., 2019**). Thus, the preservation technique of the collected bacterial cultures must ensure that the recovered starter cultures perform in the same manner as the originally isolated species (**El-Dein et al., 2022**).

One method that has commonly been used to prepare dried starter cultures for food applications is freeze-drying (**Sandhya & Disha, 2020, Guowei et al., 2019, de Melo Carvalho 2019**). In this process, dehydration of LAB imposes environmental stress on the bacterial cells, such as freezing, drying, long-term exposure to low-water activities and rehydration. Microbial survival during this process depends on many factors, including the intrinsic resistance traits of the strains, initial concentration of microorganisms, growth conditions, drying medium and protective agents, storage conditions (temperature, atmosphere, relative humidity) and rehydration conditions (**Estilarte et al., 2021, Jeantet & Jan, 2021, Wang et al., 2019, Noguerol et al., 2021**). Currently, there is little or no study that has reported on the survival performance of freeze-dried lactic acid bacteria cultures isolated from traditional Ghanaian fermented milk. Previous investigations on Ghanaian fermented milk products have focused on isolation and characterization of the predominant microorganisms to develop starter cultures for improved fermentation, food safety and quality (**Agyei et al., 2020, Motey et al., 2021, Sessou et al., 2019, Ayivi et al., 2020, Owusu-Kwarteng et al., 2020**). The purpose of this study, therefore, was to evaluate the survival rates and fermentation performance of indigenous lactic acid bacterial cultures, isolated from traditional Ghanaian fermented milk, following freeze-drying. Furthermore, the effects of different drying medium (skim milk & cassava floor) and storage condition (ambient & refrigeration) on the survival and performance of the freeze-dried LAB cultures were also determined.

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Methodology

Study Design

A schematic representation of this study is shown in Figure 1. Previously isolated identified lactic acid bacterial strains or their combinations were treated with cassava flour and skimmed milk as cryoprotectants for freeze-drying. Following freeze-drying, the cultures were stored in plastic or glass containers and stored under different temperatures (4 °C and 25 °C) over four weeks and monitored for their survival rates. Furthermore, the cultures were assessed for their fermentation performance in yoghurt production.

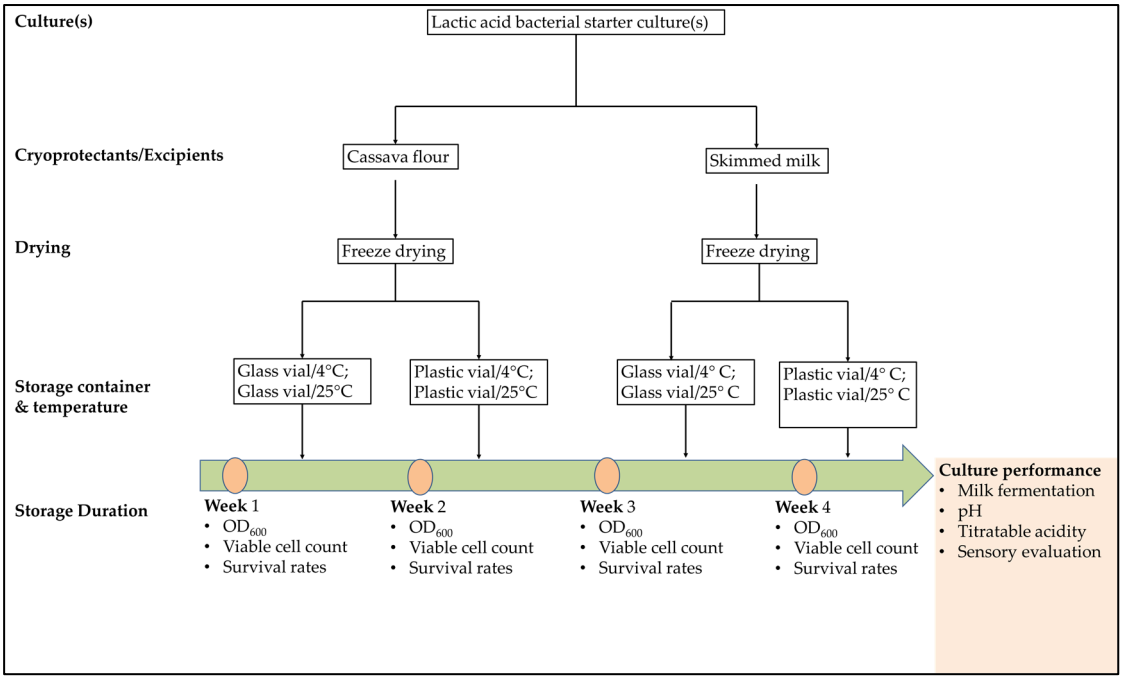

Figure 1. Study design; Each Selected Lactic Acid Bacteria: *Lactococcus lactis* (L3), *L. delbrueckii* (L12), *Leuconostoc mesenteroides* (L20), and their combinations (L3 + L12, L3 + L20, L12 + L20, L3 + L12 + L20) goes through these treatments.

Lactic acid bacterial strains

The strains used in this study include *Lactococcus lactis (L3), L. delbrueckii (L12), Leuconostoc mesenteroides* (L20) and their combinations *(*L3 + L12, L3 + L20, L12 + L20, L3 + L12 + L20*).* The strains were isolated from spontaneously fermented milk obtained from Navrongo and Accra in Ghana. The LAB strains were previously identified by sequencing of sequencing of the 16S rRNA and in MRS broth with 20% glycerol at – 20°C as stock cultures.

Preparation of Cultures for freeze-drying

Lactic acid bacterial cells were separately grown in 200 mL MRS-broth in Erlenmeyer flasks at 35 °C anaerobically for 24 h. The cells were then harvested by centrifugation (Labofuge200) at 5000rpm for 10 mins. The harvested cells were washed in phosphate buffer solution (PBS) and initial concentration (Optical Density, OD_{600}) of the cells was determined using a spectrophotometer (SM22 PC, SurgienField instrument, England).

Preparation of freeze-drying career materials

Skimmed milk powder (20 g) as an excipient was reconstituted in 100 mL of distilled water and autoclaved at 121 °C for 15 mins, and cassava flour (20 g), was oven sterilized at 160 °C for 30 mins before mixing with 100 mL distilled water. Reconstituted excipients were allowed to cool to room

 doi:10.20944/preprints202301.0173.v1

temperature. The harvested lactic acid bacterial cells were suspended in the reconstituted excipient solutions and aliquoted (500 µL) into 1.8 mL glass and plastic vials.

Freeze-drying procedure

LAB samples in glass and plastic vials were frozen in the chamber of a Telstar (Lyoquest) laboratory freeze dryer for 10 h at -55 °C. Vial caps were removed after the freezing was completed and replaced with 100% PTFE thread seal tape (ISO CERTIFIED 19MM x 0.10mm x 20M) procured from Navrongo market and holes were made on the seal using a sterilized needle procured from Navrongo market.

The LAB samples were loaded in 500 mL Erlenmeyer flask supported with cotton on the inside and held at the manifolds of the freeze-dryer and subsequently dried in the freeze-drier using the same Telstar laboratory freeze dryer under a vacuum pressure of 100mtor (1.33mbar) for 20 h. LAB Samples were disconnected from the freeze-drier and the sealed tapes were immediately replaced with the caps of the cryovials to avoid contamination. Cell viability was measured immediately after freeze-drying and this represents the initial (Day 0) of cell viability.

Storage conditions for freeze-dried cultures

The LAB samples were divided into two equal portions, with each portion containing equal samples held in a plastic and glass vials. Part of the LAB samples were stored in the refrigerator (refrigerated condition) and the other half was stored at room temperature (ambient condition). A weekly viability study was conducted on the stored LAB samples for four weeks.

Viability assays

Preparation of resuscitation media

Freeze-dried samples (10 mg) each were reconstituted in 900 µL of peptone water and vortexed to obtain a uniform mix, and 100 µL each of the reconstituted sample was then added to 5ml of prepared MRS broth and incubated at 35 °C for 24 h.

Measurement of optical density (OD)/viable cell counting

Initial cell concentration (optical density) was determined using a spectrophotometer (SM22 PC, SurgienField instrument, England) for cell viability and survivability at a wavelength of 600 nm (OD_{600}). A plastic reusable cuvette was used to hold the samples to be measured in the spectrophotometer machine. The cuvette was wiped with clean cotton soaked in ethanol before the next measurement is taking to avoid cross-contamination. OD_{600} values were measured after the first week of storage, and the experiment was repeated for three more times (once every week).

Determination of survival rates

The percentage survival of the strains after the freeze-drying process was expressed as follows:
Survival % = Ni / Nf X 100

Where N_f is the CFU/g at the end of freeze-drying and N_i is the CFU/g before freeze-drying (at the end of centrifugation) (**Yao et al. 2009**).

Performance evaluation of freeze-dried culture in milk fermentation

Inoculation of cow milk samples and fermentation

Freshly collected cow milk samples were standardized to have 4% fat and distributed into 250 mL Erlenmeyer flasks at 100 ml per flask. The milk samples were then pasteurized at 75 °C for 15 min and cooled to 35 °C (**Abrahamsen, & Narvhus, 2022**). Milk samples were inoculated with 0.01 % (w/v) of freeze-dried cultures (having a viable count of approximately 10^8 CFU/mL) according to the single and combined starter cultures shown in Table 1**.** Inoculated milk samples were incubated

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at 35 °C for 16 h (**Akabanda et al., 2014**). For spontaneous fermentation (control), fresh milk was allowed to ferment in a clean plastic container at ambient temperature for 16 h without initial sterilization of the milk.

Table 1. Single and combined freeze-dried starter cultures on fermentation.

Fermentation	Starter culture	Codes of starter culture
Single starter culture	*Lactococcus lactis*	L3
	L. delbrueckii subsp. bulgaricus	L12
	Leuconostoc mesenteroides	L20
Combined starter cultures	*Lactococcus lactis* + *L. delbrueckii*	L3+L12
	Leuconostoc mesenteroides + *L. delbrueckii*	L20+L12
Spontaneous fermentation (control)	No starter added	SPF

Bacterial growth and acidification of milk

For determination of bacterial growth during starter cultures fermentation, 10ml of fermenting samples were serially diluted (10^{-1} to 10^{-9}) using sterile phosphate buffer solution. Appropriate decimal dilutions were spread on MRS agar. After solidification, inoculated plates were incubated at 30 °C for 48 h. The colonies were counted and expressed as $\log_{10}$ CFU/ml.

To determine the performance of freeze-dried bacterial cultures by acidification properties, the pH of fermenting milk samples was determined using a digital pH meter (Crison basic 20, Barcelona), calibrated with standard buffer solutions at 30 (±2) °C. All measurements were carried out in triplicate presented as means ± standard deviations. Furthermore, titratable acidity (TA) was determined by titrating fermenting milk samples against 0.1 N sodium hydroxide (NaOH) solution and the results were expressed as % lactic acid produced.

Consumer sensory evaluation of fermented milk

Fermented milk Product (yogurt) prepared by fermentation with different freeze-dried starter cultures were served to 50 volunteers of untrained panelists (drawn from University for Development Studies and Navrongo Community) who are familiar with the traditional yogurt (**Owusu-Kwarteng et al., 2018**). In separate sensory evaluation booths, the panels independently evaluated the various products for their sensory qualities including color, odor, taste, texture, and overall acceptability using a nine-point hedonic scale with 1, 5, and 9 represent 'dislike extremely', 'neither like nor dislike', and 'like extremely', respectively. All six fermented milk products were presented to the panelists randomly placed side-by-side, with each panelist receiving 2 rounds of each product and water for rinsing their mouths. Spontaneously fermented milk (without added known starter culture) served as the control sample. Before the assessment, a detailed explanation of the process of evaluation was given to the panelists. After judging appearance, the panelists were then allowed to taste the samples and evaluate other sensory properties using the 9-point hedonic scale. The assessors were made to wash their mouth with water after evaluating each product.

Statistical analysis

All experiments were repeated at least three times and raw data generated were entered into excel spreadsheet for further processing and management. Descriptive statistics including bar charts and line (time-series) graphs were used to analyse survival rates and performance of freeze-dried LAB cultures. One-way analysis of variance (ANOVA) was used to compare the means. Means were

 doi:10.20944/preprints202301.0173.v1

separated by Tukey's family error rate multiple comparison test using the MINITAB statistical software package (MINITAB Inc. Release 14 for windows, 2004), and differences in means were considered statistically significant at $p < 0.05$.

Results

Viability of single strains Lactic Acid Bacteria after freeze-drying.

Single strains of lactic acid bacteria, *Lactococcus lactis* (L3), *Lactobacillus delbrueckii* (L12), and *Leuconostoc mesenteroides* (L20) were freeze-dried in two different kinds of excipients (cassava-CSA and skimmed milk-MLK) and in two different kinds of storage material (Plastic-P and Glass-G) to ascertain which treatment/condition best suits or support the functionality and viability of the lactic acid bacteria after a period of four weeks following freeze-drying. The Result's showed that both *Lactococcus lactis* (L3) (Figure 2) and *L. delbrueckii* (L12) (Figure 3) survived very well in cassava using plastic as the storage material (CSA_P) representing 68.87% and 70.91% respectively and *Leuconostoc mesenteroides* (L20) (Figure 3) survived well in skimmed milk under glass as the storage (MLKG) material representing 68.36% immediately after freeze-drying (control).

L. delbrueckii (L12) survived highly (Figure 3) (70.91%) when treated with cassava with plastic as the storage container as compared to the other cultures immediately after freeze-drying (Figure 3) immediately after freeze-drying (Control).

Viability of single strains lactic acid bacteria following freeze-drying after weeks of storage.

Lactococcus lactis (L3) showed superior survival rates across all the weeks (4) of storage in all the different treatments as it recorded a survival rate of between 53.4% to 76.94% under both storage temperatures. At the end of the fourth week, L3 survived better in skimmed milk using glass as storage container representing 75.45% survival rate under ambient storage temperature of 25 °C (Figure 2) at both 24 h and 48 h storage time.

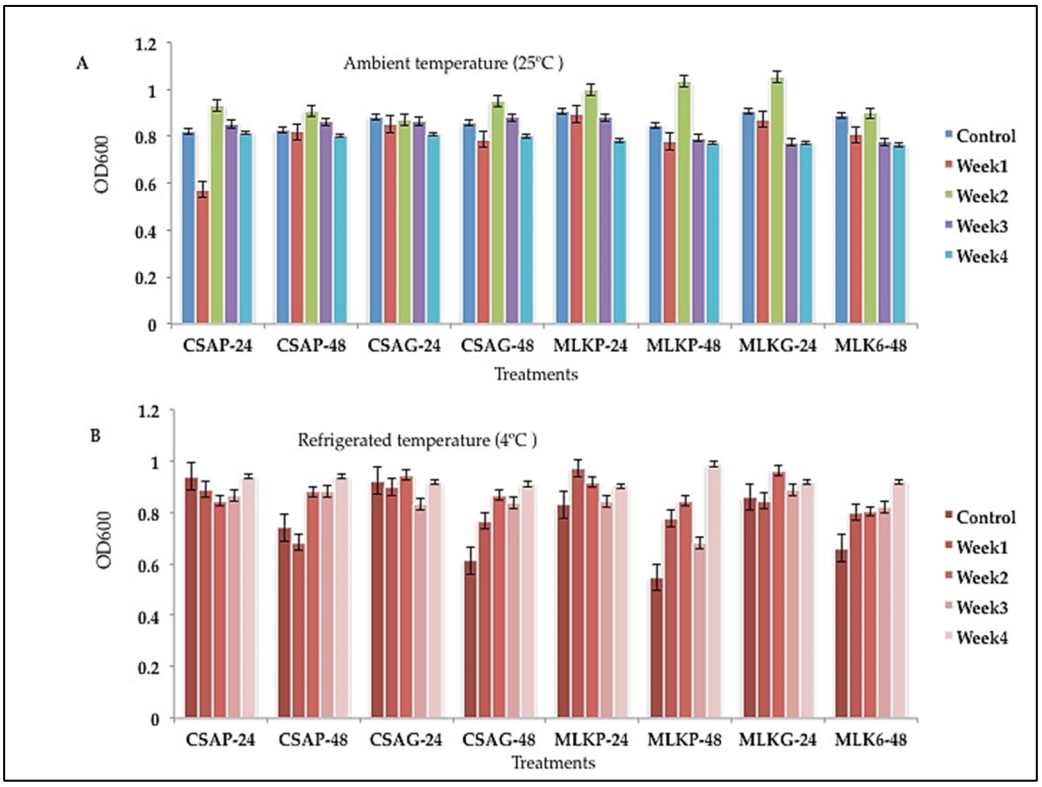

Figure 2. Viability of *Lactococcus lactis* (L3) following freeze-drying, stored at **A)** Ambient temperature (25°C), and **B)** Refrigerated temperature (4°C) for 24 h and 48 h. CSAP-24-

 doi:10.20944/preprints202301.0173.v1

Cassava in plastic at 24 h, CSAP-48-Cassava in plastic at 48 h, CSAG-24-Cassava in glass at 24 h, CSAG-48-Cassava in glass at 48 h, MLKp-24-Milk in plastic at 24 h, MLKP-48-Milk in plastic at 48 h, MLKG-24-Milk in glass at 24 h, MLKG-48-Milk in glass at 48 h. (Values represent means of three replicate experiments, ±: standard deviation).

The survival rate of *Lactobacillus delbrueckii* (L12) ranges between 62.1% to 89.45% across all the weeks of storage in all the different treatments under both storage temperatures (Figure 3 A&B). L12 survived better in the first week (89.45%) when treated with cassava under plastic storage container, and also survived better at the end of the fourth week when treated in skimmed milk using glass storage container representing 75.97% survival rate under ambient storage temperature of 25 °C (Figure 3 A).

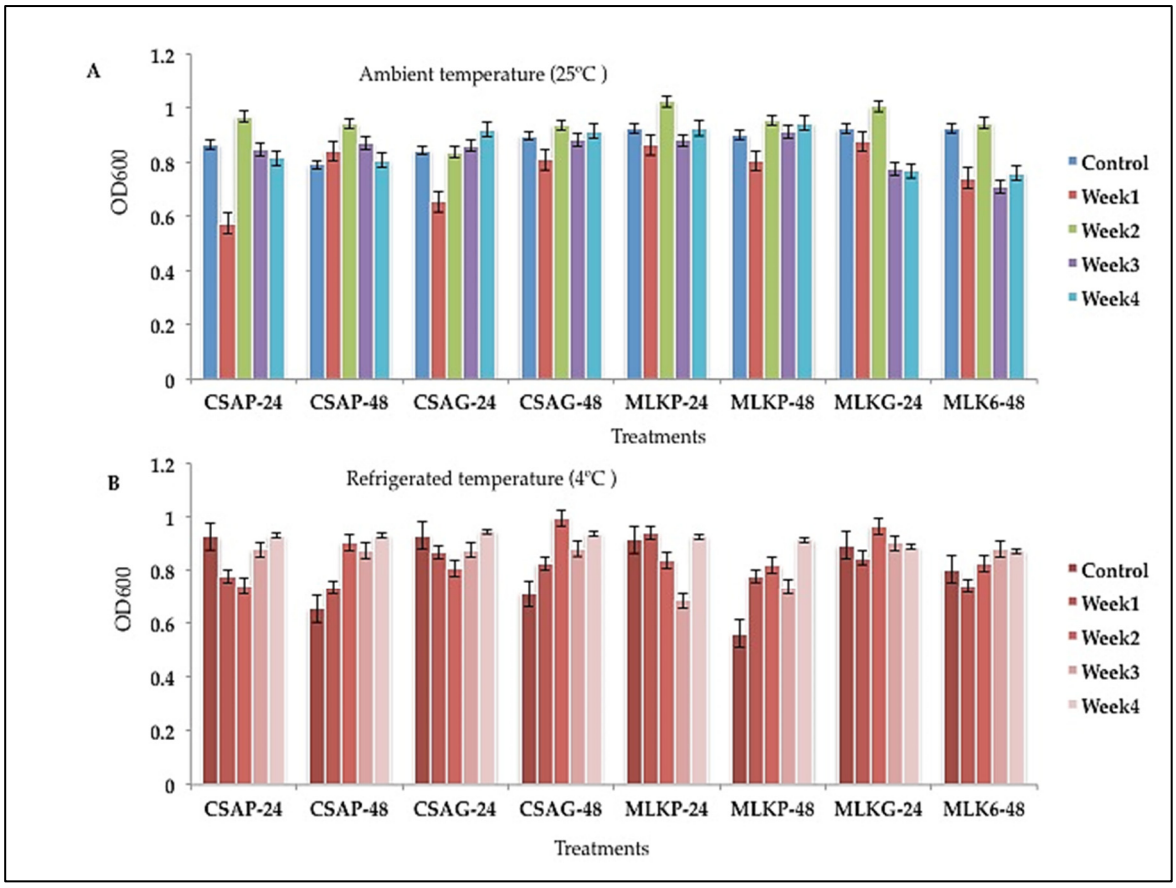

Figure 3. Viability of *Lactobacillus delbrueckii* (L12) following freeze-drying, stored at A) Ambient temperature (25°C), and B) Refrigerated temperature (4°C) for 24 h and 48 h. CSAP-24-Cassava in plastic at 24 h, CSAP-48-Cassava in plastic at 48 h, CSAG-24-Cassava in glass at 24 h, CSAG-48-Cassava in glass at 48 h, MLKp-24-Milk in plastic at 24 h, MLKP-48-Milk in plastic at 48 h, MLKG-24-Milk in glass at 24 h, MLKG-48-Milk in glass at 48 h. (Values represent means of three replicate experiments, ±: standard deviation).

The survival rate of *Leuconostoc mesenteroides (L20),* ranges between 60.59% to 89.91% across all the weeks of storage in all the different treatments under both storage temperatures (Figure 3 A&B). However, L20 survived better at the end of the fourth week when treated with cassava using plastic storage container representing 89.91% survival rate under ambient storage temperature of 25°C and 62.12% survival rate when treated with under glass storage container at refrigerated temperature of 4°C (Figure 3 A&B).

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Viability of combined strains Lactic Acid Bacteria after freeze-drying after.

Combined strains of lactic acid bacteria (Lactococcus *lactis* (L3) + *Lactobacillus delbrueckii* (L12), *Lactococcus lactis* (L3) + *Leuconostoc mesenteroides* (L20), *Lactobacillus delbruesckii subsp bulgaricus* (L12) + *Leuconostoc mesenteroides* (L20) and *Lactococcus lactis* (L3) + *Lactobacillus delbrueckii* (L12) + *Leuconostoc mesenteroides* (L20)) were also freeze-dried in two different kinds of excipients (cassava-CSA and skimmed milk-MLK) and in two different kinds of storage material (Plastic-P and Glass-G) to ascertain which treatment best supports the functionality of the lactic acid bacteria following freeze-drying. Results showed that all the different combined strains survived very well in cassava using plastic as the storage material (CSA_P) with *Lactococcus lactis* (L3) + *Lactobacillus delbruesckii* (L12) representing 69.84%, *Lactococcus lactis* (L3) + *Leuconostoc mesenteroides* (L20) representing 67.3%, *Lactobacillus delbrueckii* (L12) + *Leuconostoc mesenteroides* (L20) representing 68.21% and *Lactococcus lactis* (L3) + *Lactobacillus delbruesckii subsp bulgaricus* (L12) + *Leuconostoc mesenteroides* (L20) representing 68.19%. However, *Lactococcus lactis* (L3) + *Lactobacillus delbrueckii* (L12) was able to survive survived better (69.84%) when treated with cassava with plastic as the storage container (A) immediately after freeze-drying (control) (Figures 4–7) immediately after freeze-drying (control).

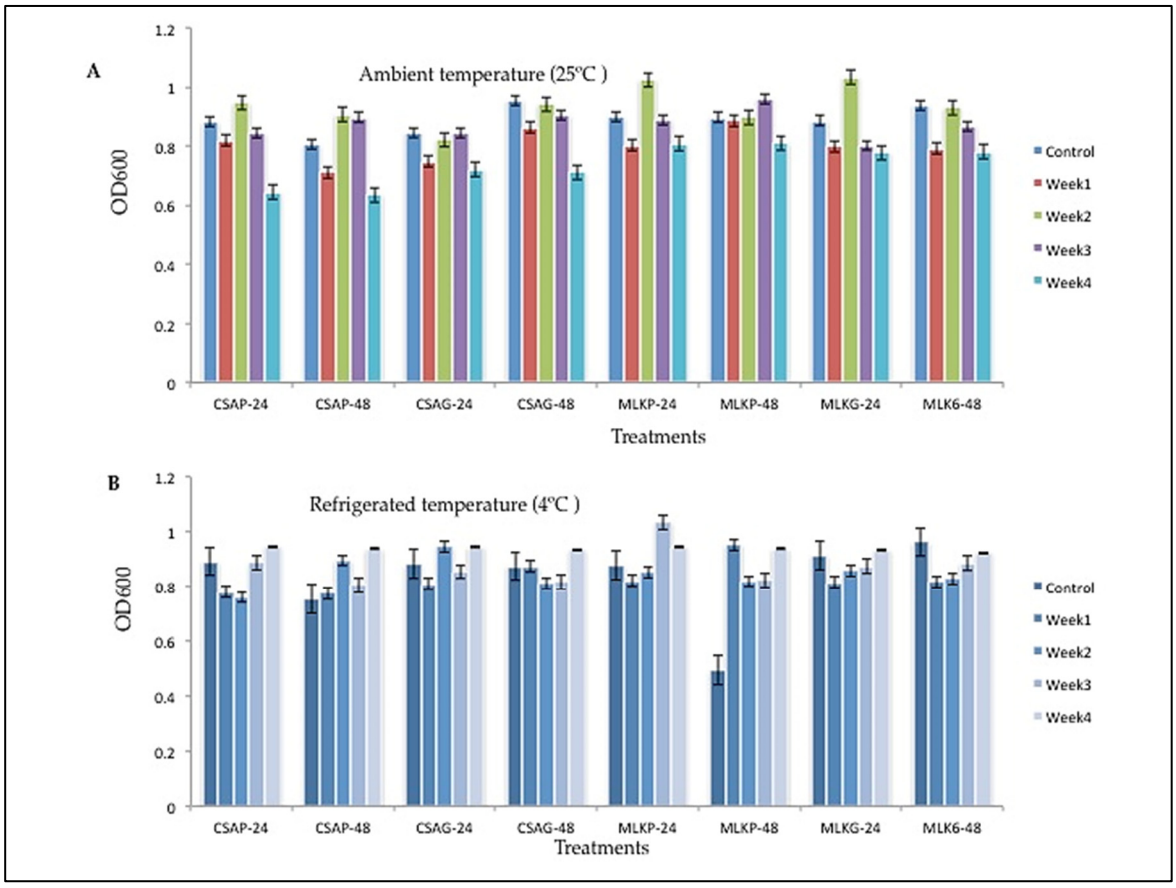

Figure 4. Viability of *Leuconostoc mesenteroides* (L20) following freeze-drying, stored at A) Ambient temperature (25°C), and B) Refrigerated temperature (4°C) for 24 h and 48 h. CSAP-24-Cassava in plastic at 24 h, CSAP-48-Cassava in plastic at 48 h, CSAG-24-Cassava in glass at 24 h, CSAG-48-Cassava in glass at 48 h, MLKp-24-Milk in plastic at 24 h, MLKP-48-Milk in plastic at 48 h, MLKG-24-Milk in glass at 24 h, MLKG-48-Milk in glass at 48 h. (Values represent means of three replicate experiments, ±: standard deviation).

Viability of combined strains Lactic Acid Bacteria following freeze-drying after weeks of storage.

The survival rate of the combined cultures of *Lactococcus lactic* (L3) *and Lactobacillus delbrueckii* (L12) ranges between 55.41% to 90.14% across all the weeks of storage in all the different treatments

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under both storage temperatures (Figure 4 A & B). However, L3 + L12 survived superiorly at the first week when treated with cassava under plastic storage container at refrigerated temperature of 4 °C (90.14%). L3 + L12 survived better at the end of the fourth week when treated in cassava using plastic storage container representing 78.81% survival rate under ambient storage temperature of 25°C and 61.73% survival rate when treated with skimmed milk under glass storage container at refrigerated temperature of 4 °C (Figure 4 A & B).

The survival rate of the combined cultures *of Lactococcus lactis* (L3) and *Leuconostoc mesenteroides* (L20), ranges between 57.03% to 78.45% across all the weeks of storage in all the different treatments under both storage temperatures (Figure 5 A and B). L3 + L20 However, survived better at the first week when treated with skimmed milk under plastic storage container at refrigerated temperature of 4°C (78.45%), and also survived better at the end of the fourth week when treated in cassava using glass storage container representing 77.28% survival rate under ambient storage temperature of 25°C and 63.96% survival rate when treated with skimmed milk under glass storage container at refrigerated temperature of 4°C (Figure 5 A and B).

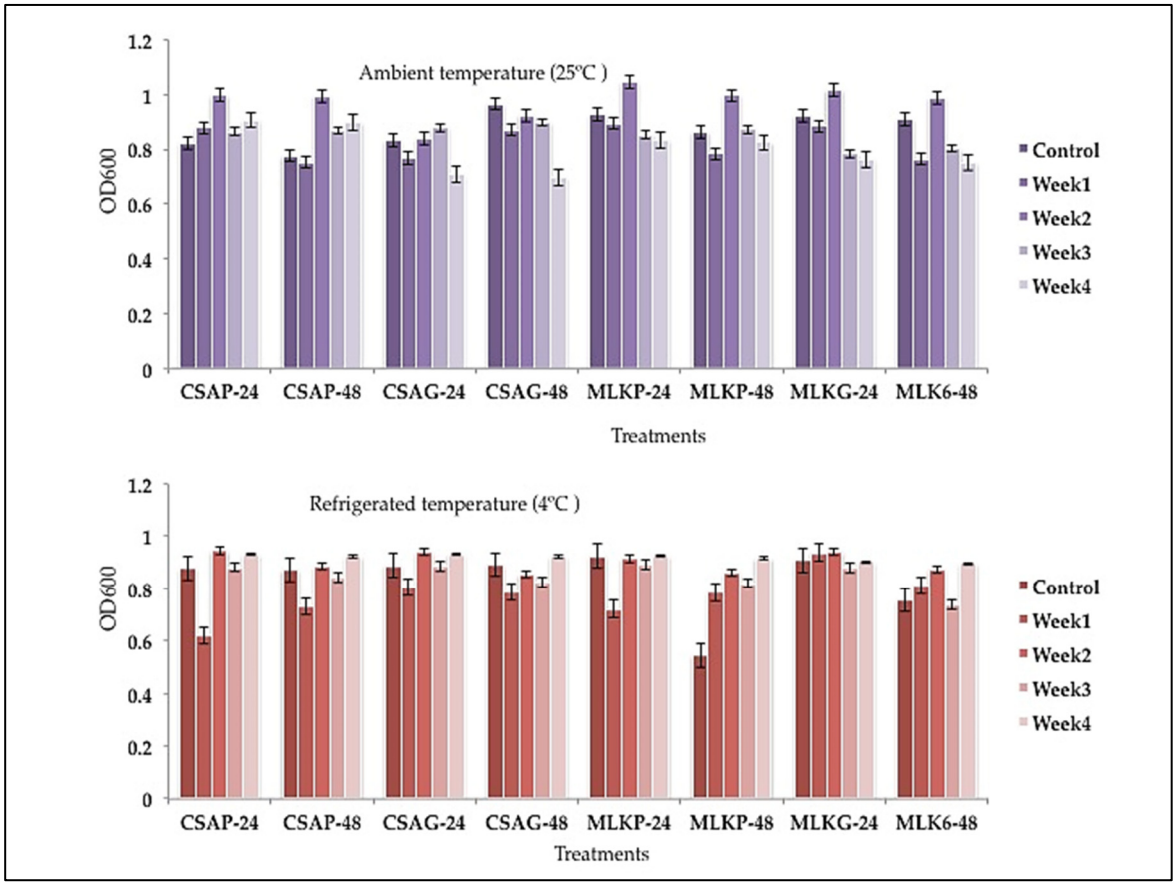

Figure 5. Viability of *Lactococcus lactis* (L3) + *Lactobacillus delbrueckii* (L12) following freeze-drying, stored at A) Ambient temperature (25°C), and B) Refrigerated temperature (4°C) for 24 h and 48 h. CSAP-24-Cassava in plastic at 24 h, CSAP-48-Cassava in plastic at 48 h, CSAG-24-Cassava in glass at 24 h, CSAG-48-Cassava in glass at 48 h, MLKp-24-Milk in plastic at 24 h, MLKP-48-Milk in plastic at 48 h, MLKG-24-Milk in glass at 24 h, MLKG-48-Milk in glass at 48 h. (Values represent means of three replicate experiments, ±: standard deviation).

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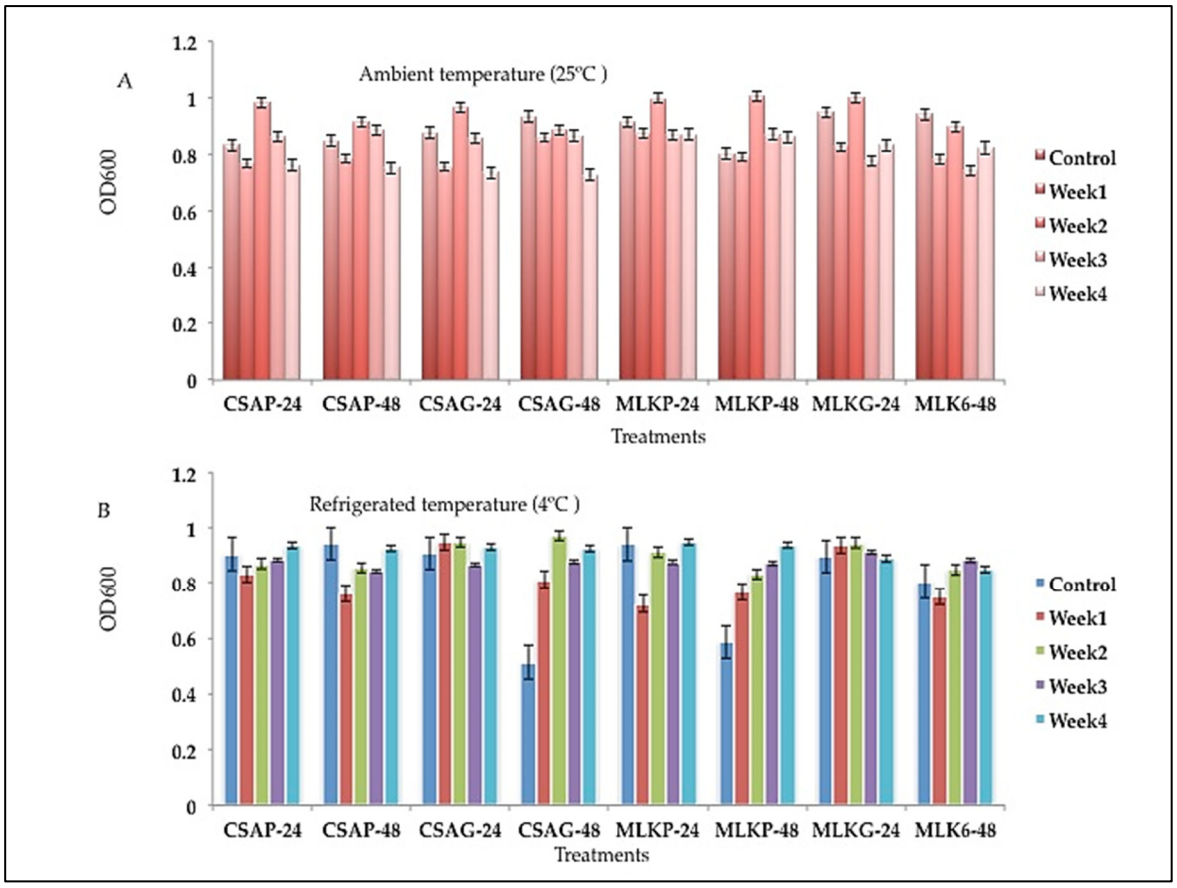

Figure 6. Viability of *Lactococcus lactis* (L3) + *Leuconostoc mesenteroides* (L20) following freeze-drying, stored at A) Ambient temperature (25 °C), and B) Refrigerated temperature (4 °C) for 24 h and 48 h. CSAP-24-Cassava in plastic at 24 h, CSAP-48-Cassava in plastic at 48 h, CSAG-24-Cassava in glass at 24 h, CSAG-48-Cassava in glass at 48 h, MLKp-24-Milk in plastic at 24 h, MLKP-48-Milk in plastic at 48 h, MLKG-24-Milk in glass at 24 h, MLKG-48-Milk in glass at 48 h. (Values represent means of three replicate experiments, ±: standard deviation).

The survival rate of the combined cultures of *Lactobacillus delbrueckii (L12)* and *Leuconostoc mesenteroides (L20*), ranges between 54.37% to 77.09% across all the weeks of storage in all the different treatments under both storage temperatures (Figure 6 A and B). L12 + L20 However, survived better at the end of the fourth week when treated in cassava using glass storage container representing 77.09% survival rate under ambient storage temperature of 25°C and 77.30% survival rate when treated with skimmed milk under plastic storage container at refrigerated temperature of 4°C (Figure 6 A and B).

The survival rate of the combined cultures of *Lactococcus lactis (L3), Lactobacillus delbrueckii (L12)* and *Leuconostoc mesenteroides* (L20) ranges between 52.62% to 81.72% across all the weeks of storage in all the different treatments under both storage temperatures (Figure 7 A and B). L3 + L12 + L20 However, survived better at the end of the fourth week when treated in cassava using glass storage container representing 81.72% and 70.00% survival rate under ambient storage temperature of 25°C and 70.00% survival rate when treated with skimmed milk under plastic storage container at refrigerated temperature of 4°C (Figure 7 A and B).

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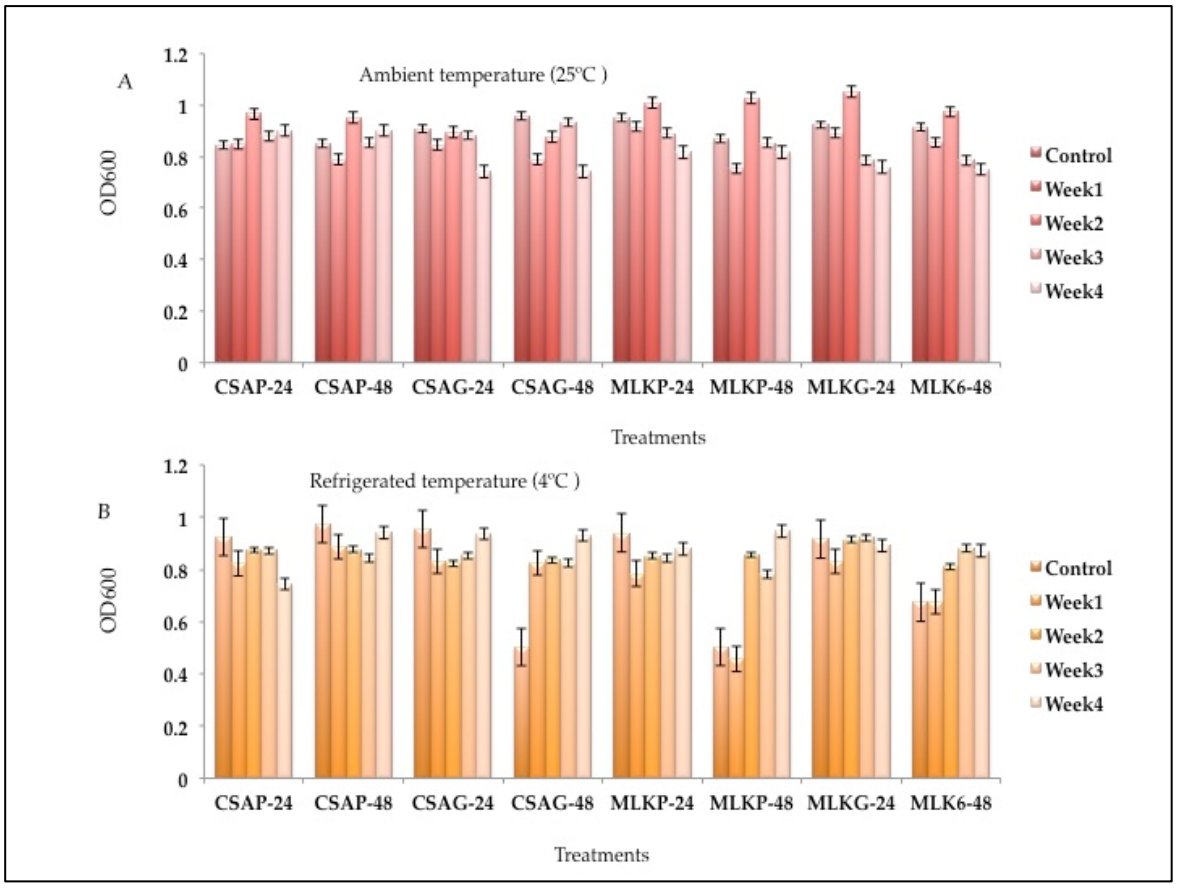

Figure 7. Viability of *Lactobacillus delbrueckii* (L12) + *Leuconostoc mesenteroides* (L20) following freeze-drying, stored at A) Ambient temperature (25°C), and B) Refrigerated temperature (4°C) for 24 h and 48 h. CSAP-24-Cassava in plastic at 24 h, CSAP-48-Cassava in plastic at 48 h, CSAG-24-Cassava in glass at 24 h, CSAG-48-Cassava in glass at 48 h, MLKp-24-Milk in plastic at 24 h, MLKP-48-Milk in plastic at 48 h, MLKG-24-Milk in glass at 24 h, MLKG-48-Milk in glass at 48 h (Values represent means of three replicate experiments, ±: standard deviation).

Performance of freeze-dried LAB starter cultures during milk fermentation

For the performance of freeze-dried cultures, total lactic acid bacterial count and pH were determined during milk fermentation. Generally, LAB counts increased with fermentation time while pH decreased (Figure 8). *Lactococcus lactis* recoded the lowest pH rate as well as the highest CFU/mL while yogurt fermented from the spontaneous fermentation of milk recorded the highest pH value as well as the lowest CFU/mL at the end of the fermentation period.

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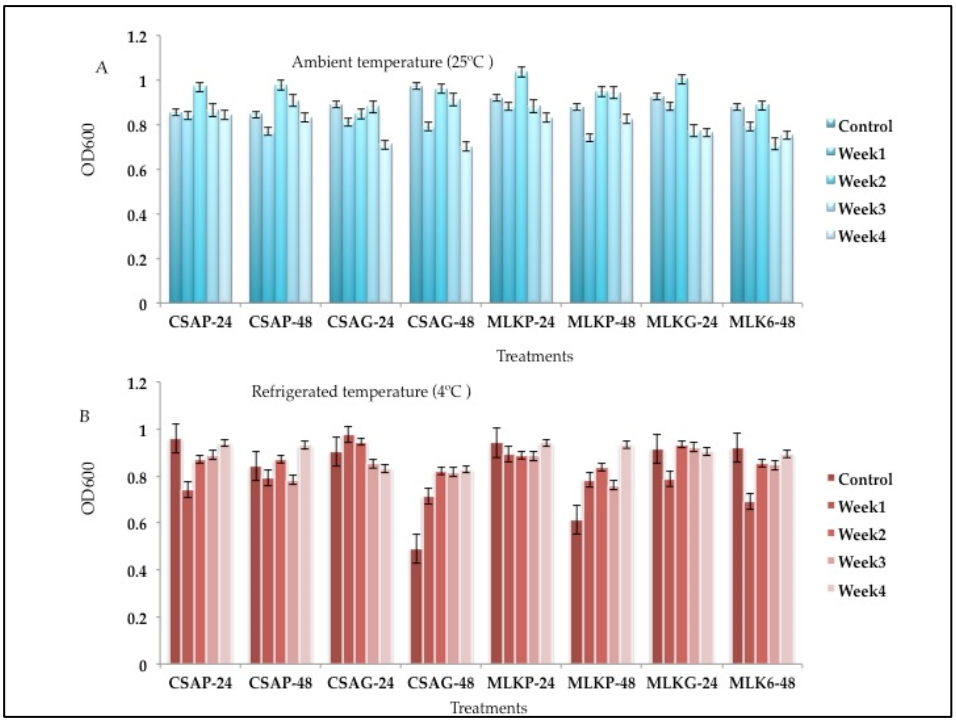

Figure 8. Viability of *Lactococcus lactis* (L3) + *Lactobacillus delbrueckii* (L12) + *Leuconostoc mesenteroides* (L20) following freeze-drying, stored at A) Ambient temperature (25°C), and B) Refrigerated temperature (4 °C) for 24 h and 48 h. CSAP-24-Cassava in plastic at 24 h, CSAP-48-Cassava in plastic at 48 h, CSAG-24-Cassava in glass at 24 h, CSAG-48-Cassava in glass at 48 h, MLKp-24-Milk in plastic at 24 h, MLKP-48-Milk in plastic at 48 h, MLKG-24-Milk in glass at 24 h, MLKG-48-Milk in glass at 48 h. (Values represent means of three replicate experiments, ±: standard deviation).

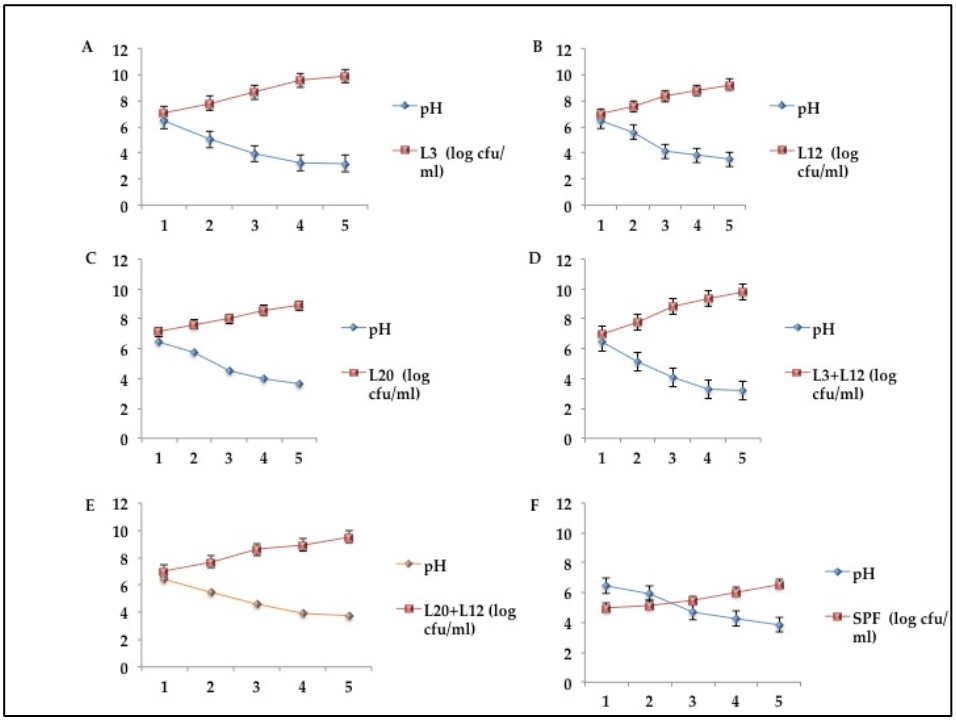

Figure 9. LAB count (log cfu/ml) and pH of yogurt produced from freeze-dried starter cultures.

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Consumer sensory evaluation of yogurts fermented with freeze-dried starter culture

The effect of freeze-dried LAB cultures on consumer sensory attribute was evaluated using a nine-point hedonic scale with 1, 5, and 9 is presented in Table 1. The type of starter culture did not have significant effect on colour of yogurt. However, significant difference ($P < 0.05$) was observed among the different starter culture on yogurt odour, taste, texture, and overall acceptability. The combined starter culture of *Lactococcus lactis (L3)* and *Lactobacillus delbrueckii* (L12), and a single starter of *Leuconostoc mesenteroides* (L20) were highly scored for odour, taste and texture. For overall acceptability, consumers scored yogurts produced with combined starter culture of *Lactococcus lactis (L3)* and *Lactobacillus delbrueckii* (L12), or single culture of only *Lactococcus lactis* as the most preferred products. Except colour where no significant difference was observed, yogurt produced by spontaneous fermentation was least preferred in all other sensory attributes (Table 2). Also, there was no significant difference between all the strains (single and combined) including yogurt produced from spontaneous fermentation regarding appearance (color). For product odor, taste as well as texture, the combined strains of L3 + L12 is significantly higher as compared to the other strains, except for L3 which showed no significant difference compared to L3 + L12 regarding taste ($P < 0.05$). Nonetheless, yogurt produced with a single starter culture of *Lactococcus lactis* (L3) or combined starter cultures of *Lactococcus lactis* and *Lactobacillus delbrueckii* (L3 + L12) showed significantly higher overall acceptability.

Table 2. Consumer sensory evaluation of traditional yogurts produced with freeze-dried starter cultures.

Starter culture	Sensory attribute				
	Colour	**Odour**	**Taste**	**Texture**	**Overall acceptability**
L3	8.04 ± 1.35^{a}	7.16 ± 1.21^{a}	7.23 ± 1.30^{a}	7.72 ± 1.52^{a}	8.36 ± 1.18^{a}
L12	7.95 ± 1.40^{a}	7.01 ± 1.19^{a}	6.65 ± 1.25^{b}	7.60 ± 1.45^{a}	7.22 ± 1.15^{b}
L20	8.00 ± 1.20^{a}	8.37 ± 1.25^{b}	7.14 ± 1.30^{ac}	7.84 ± 1.33^{ab}	7.39 ± 1.50^{b}
L3+L12	8.06 ± 0.95^{a}	8.83 ± 1.10^{b}	7.37 ± 1.05^{a}	8.08 ± 0.96^{b}	8.45 ± 0.99^{a}
L20+L12	7.90 ± 1.15^{a}	7.20 ± 1.21^{a}	6.91 ± 1.27^{c}	7.75 ± 1.65^{a}	7.30 ± 1.05^{b}
SPF	7.96 ± 1.20^{a}	6.03 ± 0.83^{c}	6.20 ± 1.24^{d}	7.02 ± 0.73^{c}	6.25 ± 1.00^{c}

Values represent means of three independent experiments, ±: standard deviation. Values in the same column with different superscript letters are significantly different from each other ($P < 0.05$). L3: *Lactococcus lactis*; L12: *Lactobacillus delbrueckii*; L20: *Leuconostoc mesenteroides*; L3+L20: combined starter of *Lact. lactis* and *Leuc. mesenteroides*; L20+L12: combined starter of *Leuc. mesenteroides* and *Lact. lactis*; SPF: spontaneous fermentation (without starter culture).

Discussion

Lactic acid bacteria are predominantly used in the fermentation of milk into fermented milk products, often referred to us yogurt starter cultures or simply starter cultures (**Ahmad et al., 2020, Kumar et al 2020, Hwang eta al., 2018, Abesinghe et al., 2019**). The purpose of this study was to freeze-dry lactic acid bacteria cultures, determine the (viability) survival rates after freeze-drying and under the influence of different storage materials such as glass and plastic and in different cryoprotectants/buffers/excipients such as skimmed milk and cassava. Also *Lactococcus lactis (L3), Lactobacillus delbrueckii (L12)*, and *Leuconostoc mesenteroides (L20)* and their combinations, *Lactococcus lactis (L3) + Lactobacillus delbrueckii subsp bulgaricus (L12), Lactococcus lactis (L3) + Leuconostoc mesenteroides (L20), Lactobacillus delbrueckii subsp bulgaricus (L12) +Leuconostoc mesenteroides (L20)* and

 doi:10.20944/preprints202301.0173.v1

Lactococcus lactis (L3) + Lactobacillus delbrueckii subsp bulgaricus (L12) + Leuconostoc mesenteroides (L20) were the lactic acid bacteria used for the purpose of this study as they are the most common kind of starter cultures used in yogurt production across Africa (**Gu et al., 2021, Celik & Temiz, 2022, Arab et al., 2022, Ghosh 2019**).

It was observed in the course of the studies that, the strains performed better under the ambient storage temperature at the fourth week This observation is of immense interest as we are primarily trying to preserve microbial cultures for a longer time period (4 weeks in this study) while minimizing cost in the powdered state. Therefore, the ability of the strains to perform well at ambient condition at the fourth week suggest that within the limits of this study, the strains/cultures would not need refrigeration over 4 weeks' storage to be use as viable starter cultures. This will reduce to a large extent the cost of maintaining these cultures. Also, transportation of cultures will be less hectic as there will be no need carrying cultures under refrigeration.

Freeze-drying proved to be effective in achieving high survival rates as all the three strains and their combinations achieved >50% survival rates. Microbial preservation by freeze-drying is known to preserve the viability of cultures over long durations (**Yao et al. 2008**). Our results agree with those reported by **Yao et al. (2009)**, who found very high survival rates after freeze-drying the strains of *L. Plantarum* VE36, G2/25 and *L. pentosus* LB61 with survival rates of 97.3%, 79.9%, and 76.7% respectively. The high survival rate in this study may be attributed to the quality of the sterilization techniques adopted, the efficiency of the freeze-dryer itself and or, the choice of the excipients used (cassava flour and skimmed milk powder) as good excipients can greatly impact survival rates of bacteria survival (**Berner and Viernstein 2006**). Not all microorganisms can be successfully freeze-dried especially mutants with deficient membranes, several reports have shown lactic acid bacteria been successfully freeze-dried (**Yao et al. 2009, Ambros et al., 2018, Fonseca et al., 2021, Enache et al., 2020**) with different freeze-drying approaches. This may be due to the removal of the most sensitive parts of the cell population during the freeze-drying process and low surface area of the selected strains. The high survival rates may also be attributed to the rehydration method adopted in this study, as there is an increase in survival rate when the rehydration process is slowed (**Carvalho et al. 2004).** It may also be due to the high initial cell concentration of the microorganisms, the growth conditions, and the growth media.

Many studies have not been done on the viability of lactic acid bacteria with regards to the influence of storage temperatures, excipients, and storage containers on the bacteria. Our studies showed that all the three strains and their combinations were not affected greatly by the storage containers as well as the storage temperature as all strains survived above 50%. Yao et al. (**2009**) equally recorded above 50% survival rates of 12 LAB strains out of 16 LAB strains, which they subjected to freeze-drying. The excipients (skimmed milk and cassava flour) were both able to protect the cells from deteriorating and loss of viability. Freeze-dried cultures should be stored in glass containers as very long storage time can cause atmospheric water to diffuse into plastic tubes and damage freeze-dried samples (**Bacteria freeze-drying protocol, opsdiagnostics, 2015**), even though our studies showed that samples stored in plastic containers showed very good survival rate over the four weeks' storage duration. Both excipients used in this study supported the viability of the strains. However, strains treated with cassava flour showed superior survival rate over strains treated with skimmed milk as indicated by the results above, this may be due to cassava being a polysaccharide (exopolysaccharide) with some sort of biological essence. Exopolysaccharides have two types of secreted polysaccharides with the first type (capsular polysaccharide) attached to the cell wall as a capsule and the second type (Slime exopolysaccharide) is produced as a loose unattached material.

Exopolysaccharides prevents microbial cells from desiccation, phagocytosis, phage attachment, antibiotics, toxic compounds and osmotic stress (**Degeest et al., 2001, Akabanda et al. 2014).** Nevertheless, due to the particulate (rough) nature of the reconstituted cassava flour, it was extremely difficult and time-consuming in micro pipetting as observed in this study, even though it was very suitable in terms of cost and durability compared to skimmed milk powder which can easily be contaminated. Skimmed milk is usually selected when it comes to industrial or scale-up or

 doi:10.20944/preprints202301.0173.v1

commercial production of freeze-dried lactic acid bacteria. This may be due to several factors such as prevention of cellular injury by stabilizing the cell membrane, creating of a porous structure in the freeze-dried product that makes rehydration easier and finally, it contains proteins that provide a protective coating for the cells (**Teixeira and Kirby 1996, Carvalho et al. 2002**). This is evident in our study as both excipients led to high survival rates of the LAB strains constituents

Our studies have shown that the bacteria strains used can survive under both ambient (25°C) and refrigerated (4°C) conditions, however, some studies have suggested that freeze-dried samples should be stored in environments with lower temperatures as it impacts survival rates for long storage of samples. In addition to temperature, relative humidity, and exposure to light, all impact survival of freeze-dried samples (**Mofidi et al. 2002**). Therefore, freeze-dried samples are suggested to be store in a relatively balanced environment, and samples should never be stored in temperatures above 30°C, samples are also suggested to be stored under vacuum and exposed to darkness (**Tiradentes et al., 2011**).

In the development of starter cultures for the production of fermented milk products, quick acidification is a topmost priority (**Yamauchi et al, 2019**). The acidification rates varied among the freeze-dried LAB strains tested. Akabanda et al. (**2014**) reported that *Lactobacillus helveticus*, *L. fermentum*, *L. plantarum*, and *L. mesenteroides* isolated from nunu were the fastest acid producers in comparison to the other strains used in their study. From the results obtained, all the selected strains tested showed a fast rate of acidification during the period of fermentation. A decrease in pH is essential in yogurt production as it accelerates coagulation and mitigation of pathogenic microflora that might invade the products (**Yamauchi et al, 2019, Körzendörfer et al., 2019, Delgado-Fernández et al., 2020)**. The selected freeze-dried strains are therefore good candidates as starter cultures for the dairy fermentation process. LAB starter cultures that possess the ability to rapidly and completely degrade lactose to lactic acid with minimal nutritional levels are generally desirable. Accelerated acidification of the raw materials means the prevention of the growth and action of undesirable microorganisms on fermented products. This has a positive impact on the aroma, texture, and flavour of the end product. A rapid decrease in pH to <4 indicates the fastest growth and inhibition of starter cultures against pathogenic microbes especially Salmonella spp (**Park and Marth 1972**).

The purpose of the study was to obtain viable LAB cultures that could be stored for over a long period using freeze-drying preservation technology. Higher colony-forming units indicate high viability and vice versa. Even though the LAB cultures survived fairly well at the end of freeze-drying and following storage over four weeks, the viability still needed to be confirmed by their ability to ferment milk at the end of the fourth week after freeze-drying. Hence, we subjected the LAB to the fermentation of milk (to produce yogurt) for 12 hours measuring cell growth and viability for every 3 hours.

Consumer sensory analysis showed varying degrees of acceptability for yogurt fermented with the different starter cultures Generally, Yogurt fermented with freeze-dried lactic acid bacteria cultures, either single or combined strains, showed improved acceptability as compared to the spontaneously fermented yogurt. The high acceptability of yogurt fermented with *Lactococcus lactis (L3)* and the combined cultures of *Lactococcus lactis* and *lactobacillus delbrueckii* (L3 + L12) could be due to the cultures being able to reduce the pH of the milk from 6.45-3.18 and 6.44-3.20 respectively. Reduction in pH is critical as it affects the organoleptic properties of the yogurt. This may be due to the fact that these cultures were able to produce the lowest pH values (high acidity) during the yogurt fermentation at the end of 12 hours. Park et al. recorded unpleasant acid taste with yogurt acidity more than 1.8% and a titratable acidity about 1.15% considered as the average (**Park et al., 2005**).

Conclusion

In this study, the potential of the three pre-selected LAB cultures (*Lactococcus lactis, Lactobacillus delbrueckii* and *Leuconostoc mesenteroides*) for use as suitable freeze-dried starter cultures for milk fermentation during yogurt production was assessed. In general, survival rates for lactic acid bacterial cultures ranged between 60.11% and 70.91% following freeze-drying. For single cultures,

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the highest survival was recorded for *Lactobacillus delbrueckii* (L12), whereas for combined the highest survival was observed for *Lactococcus lactis* (L3) combined with *Lactobacillus delbrueckii* (L12). The different excipients (cassava and milk) have different varying effects on the different bacterial cultures. All freeze-dried lactic acid bacteria starter cultures whether single or in combinations grew rapidly during yogurt making, reducing the pH of milk to below 4 units within 9 hours of fermentation. On the other hand, spontaneous fermentation (without starter cultures) was characterized by slower acidification. For overall consumer acceptability, yogurts produced with combined starter culture of *Lactoccus lactis* and *Lactobacillus delbrueckii* or single culture of *Lactococcus lactis* were the most preferred products.

Overall, *Lactococcus lactis* and *Lactobacillus delbrueckii* can be used as freeze-dried lactic acid bacterial starter culture with high survival rates and high consumer acceptability in the production of yogurt. This can be adopted for large-scale production and commercialization of yogurt production. Notwithstanding, further studies on the effects of freeze-drying and long-term storage on survival and performance of selected LAB cultures are recommended.

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