Original Research Article

Storage study of foxtail millet probiotic powder enriched with high protein and fibre

ABSTRACT

Aims: The study aimed to evaluate the storage stability of foxtail millet probiotic powder enriched with high protein and dietary fiber under two storage conditions: room temperature (27 ± 1°C) and refrigeration temperature (7 ± 1°C).
Study design: This was an experimental, laboratory-based study.
Place and Duration of Study: The study was conducted at the Department of Dairy Microbiology, Dairy Science College, Hebbal, Bengaluru, Karnataka, India, between January 2024 and October 2024.
Methodology: Foxtail millet probiotic powder enriched with high protein and dietary fiber was stored in polypropylene sachets under two storage conditions: room temperature and refrigeration temperature. Samples were systematically drawn at 7-day intervals and analyzed for key quality parameters, including probiotic viability, microbial contaminants, water activity, moisture content, alcoholic acidity, and pH. Statistical analyses were performed using Analysis of Variance (ANOVA), with a 5% significance level. A critical difference (CD) value was employed to determine the statistical significance of observed variations between treatments over the storage period.
Results: The storage stability of the optimized foxtail millet probiotic dry mix was assessed at both room temperature (27 ± 1°C) and refrigeration (7 ± 1°C) until spoilage or decline in probiotic viability below the threshold value. Probiotic viability declined significantly under both conditions, with a reduction from 11.04 $\log_{10}$ CFU/g to 8.88 $\log_{10}$ CFU/g at room temperature and 8.81 $\log_{10}$ CFU/g under refrigeration. Water activity and moisture content increased over time, reflecting the hygroscopic nature of the mix, while alcoholic acidity rose significantly in both storage conditions. Despite these changes, the mix maintained probiotic viability above the therapeutic threshold (9 $\log_{10}$ CFU/g) for 84 days at room temperature and 105 days under refrigeration, demonstrating its potential as a stable functional food product.
Conclusion: The storage study of foxtail millet probiotic powder demonstrated its stability under both refrigeration and ambient conditions, with probiotic viability exceeding the 1 billion CFU/g threshold for up to 105 days and 84 days, respectively. Significant changes in water activity, moisture content, alcoholic acidity, and pH were observed, highlighting the product's dynamic response to storage conditions.These results support the feasibility of developing shelf-stable, high-protein, high-fiber probiotic millet powders for the health-conscious market.

Keywords: Foxtail millet, Probiotics, Whey Protein Concentrate, Chia flour, Functional product, Protein enrichment, Fibre enrichment, Storage Stability, Shelf-life

1. INTRODUCTION

The increasing demand for functional foods has driven the development of nutrient-rich products that combine health benefits with extended shelf stability. Among these, millet-based formulations have gained significant attention due to their inherent nutritional qualities, including high protein and fibre content, as well as their potential for probiotication. Foxtail

millet stands out as a versatile ingredient due to its high protein content and dietary fibre (Santhosh et al., 2024a), making it an ideal candidate for probiotic-based functional food development (Santhosh et al., 2024b)

Storage stability is a critical factor influencing the quality, safety, and functionality of probiotic and millet-based formulations. Yadagouda and Ravindra (2022) demonstrated the robustness of a foxtail millet composite mix against microbial spoilage during a one-month storage period at ambient conditions, highlighting the potential of millet-based products in maintaining microbial stability. However, the viability of probiotics in such formulations is often compromised over time. Borges *et al.* (2016) observed a reduction in *Lactiplantibacillus plantarum* viability in apple powder, emphasizing the importance of optimizing storage conditions to preserve probiotic efficacy.

Several studies have explored strategies to enhance probiotic survival during storage. Bagad *et al.* (2017) reported higher probiotic viability in formulations stored at refrigeration temperatures (4°C), particularly when excipients such as sorbitol and ascorbic acid were included. These findings underscore the critical role of temperature and formulation additives in maintaining probiotic stability.

Environmental factors such as moisture content, water activity, and pH also play a significant role in the shelf life of millet-based products. Shobha *et al.* (2023) documented a substantial increase in moisture content and alcoholic acidity in white finger millet flour stored at ambient conditions, reflecting the impact of storage environments on product quality. Similarly, Goyal *et al*. (2017) noted a pH decline in pearl millet flour during storage, which could affect sensory properties and consumer acceptability.

This study investigates the storage stability of a foxtail millet probiotic powder enriched with high protein and fibre content under ambient and refrigeration conditions. Key quality parameters, including probiotic viability, water activity, moisture content, alcoholic acidity, and pH, were monitored over time. The findings aim to provide insights into the dynamic interactions between formulation properties and storage conditions, offering valuable information for optimizing the shelf life of millet-based probiotic functional foods.

2. MATERIAL AND METHODS

2.1 Materials

Foxtail Millet (*Setaria italica)*, whey protein concentrate, chia seeds and a mixed probiotic culture containing *Lactobacillus acidophilus* LA-5*, Bifidobacterium bifidum* BB-12 and *Streptococcus thermophilus.*

2.2 Preparation of foxtail millet probiotic powder enriched with high protein and fibre (Foxatil millet probiotic dry mix)

Foxtail millet (*Setaria italica*) was prepared using a systematic process to enhance its nutritional profile and suitability for probiotic enrichment. The millet was initially soaked in potable water at a millet-to-water ratio of 1:2 to promote hydration. The soaking process was conducted at 30°C for 24 hours under controlled conditions, following the methodology outlined by Santhosh *et al.* (2024c). After soaking, the water was drained, and the hydrated millet was tied in sterile muslin cloth to facilitate germination.

The germination process was carried out at 30°C for 48 hours in a controlled environment using an incubator. This step aimed to enhance the bioavailability of nutrients and reduce antinutritional factors. Post-germination, the millet was dried at 30°C until it reached a final moisture content of 5–7%. The drying process was carefully monitored to ensure uniform moisture reduction while maintaining the nutritional integrity of the millet grains. The dried millet was milled into fine flour using a milling apparatus and subsequently sieved through a 150-micron mesh to achieve uniform particle size, ensuring consistency in texture (Santhosh *et al*., 2024d).

The sieved flour was transferred to sterilized conical flasks and subjected to autoclaving at 121°C for 15 minutes under 15 psi pressure to ensure microbial safety and stability. Chia seeds, were ground into fine powder and similarly sterilized by autoclaving at 121°C for 15 minutes under 15 psi pressure. The whey protein concentrate (WPC) was sterilized using ultraviolet (UV) light. The WPC was exposed to a UV lamp with an intensity of 100 μWcm^{-2} for 60 minutes, delivering a UV-C dose of 3.6×10^{6} Jm^{-2}. This process ensured the microbial safety of WPC while preserving its functional properties (Santhosh *et al.,* 2024b).

All the prepared ingredients were combined in the specific quantities prescribed by Santhosh *et al.* (2024b) to formulate the foxtail millet probiotic powder enriched with high protein and dietary fiber.

2.3 Storage stability of optimized foxtail millet probiotic dry mix

The probiotic, protein and fibre optimized foxtail millet dry mix was stored at refrigeration temperature (7±1°C) and room temperature (27±1°C). The samples were drawn from the stored samples and were analysed for microbiological (viability and contaminants) and chemical parameters (moisture content, pH, alcoholic acidity and water activity) and at an interval of 7 days for samples stored at both room temperature and refrigerated condition until the spoilage is observed or probiotic viability is maintained.

2.3.1 Microbiological testing for the viability of probiotics

To prepare the first dilution, 11.0 g of the sample was weighed and added to 99.0 mL of sterile phosphate buffer. Serial dilutions up to 10^{-7}, 10^{-8}, and 10^{-9} were prepared from the initial dilution. These dilutions were transferred to marked sterile petri plates. Approximately 10–15 mL of sterile de Man, Rogosa, and Sharpe (MRS) agar, maintained at 45°C, was poured into the plates, which were then allowed to solidify.

Once solidified, the plates were inverted and placed in an anaerobic candle jar for incubation at 37°C for 72 hours. After the incubation period, colonies on the MRS agar were counted. The average count from plates with colony ranges of 30 to 300 was reported as $\log_{10}$ CFU/g. Sterilized millet flour without added probiotic cultures was used as the control (Harrigan, 1998).

2.3.2 Microbiological testing for the presence of contaminants

2.3.2.1 Enumeration of aerobic spores

Using a sterile mortar and pestle, 11.0g of the sample was triturated using sterile phosphate buffer. To create a 1:10 dilution, it was then added to 99.0ml of sterile phosphate buffer. After being heated to 80℃ for 10 minutes in a water bath, the first dilution was cooled to less than 10℃. Then the needed dilutions were prepared serially by the first dilution.

Serially diluted samples were then transferred to sterile petri plates that had been labelled. Molten sterile 2% nutrient agar (NA) maintained at 45℃ was poured into marked petri dishes and allowed to solidify. By inverting the plates, all the poured plates were incubated at 37℃ for 24 to 48h. The number of nutrient agar colonies was counted after the incubation time. The average count of the countable plates which ranged from 30 to 300 were reported as $\log_{10}$ CFU/g (Harrigan, 1998).

2.3.2.2 Enumeration of yeast and mold

About 99.0ml of sterile phosphate buffer was mixed with 11.0g of the weighed samples to prepare the first dilution. The first dilution was then used to prepare the subsequent dilutions in a sequential manner.

Samples that had been serially diluted were thereafter transferred to labelled sterile petri dishes. Sterile malt extract agar (MEA) kept at 45°C was poured into designated petri plates and were allowed to solidify. All of the poured plates were incubated at 30°C for 3-5 days by inverting the plates. Following the incubation period, the number of malt extract agar colonies was counted. The average of countable plate which varied from 30 to 300, was expressed as $\log_{10}$ CFU/g (Harrigan, 1998).

2.3.3 Determination of water activity

The water activity was measured using a LabSwift-a_w equipment in the rheology lab at the Department of Dairy Technology, Dairy Science College, Hebbal, Bengaluru. It was created especially to ascertain the percentage of free water present in a test sample. The sample's water activity, represented by the a_w value, falls between 0 (absolute dryness) and 1 (condensing humidity). By entirely adding sample to the sample cup without leaving any gaps, the measurement was initiated by pressing the start/stop button. As soon as the steady value was shown, the result was read (Jayashree *et al*., 2024).

2.3.4 Determination of moisture content

5.0g sample was taken and placed in a dish that had been previously dried, weighed along with lid. The dish was then placed in oven at 130±3°C for 2h. After that, the dish was placed in a desiccator with a lid on it and allowed to cool to room temperature (25±3℃). Then it was weighed once again (FSSAI 03.005:2022).

$$\text{Moisture (\%)} = \frac{W_1 - W_2}{W_1 - W} \times 100$$

Where, W = Mass in g of the empty dish
W_1 = Mass in g of the dish with the test portion before drying
W_2 = Mass in g of the dish with the material after drying

2.3.5 Determination of alcoholic acidity

A conical flask containing 5g of sample was filled with 50ml of neutral 90% ethyl alcohol. The mixture was allowed to stand for 24h on occasion swirling and then filtered through Whatman filter paper. Using phenolphthalein as an indicator, 10ml of filtrate was further titrated with standard 0.05N NaOH solution to the pink end point (FSSAI 03.014:2022).

$$\text{Alcoholic acidity (with 90\% alcohol) (\% by mass on dry weight basis)} = \frac{24.52 \times A \times N}{M}$$

Where, A = Titre value
N = Normality of NaOH
M = Mass of sample (dry weight basis)

2.3.6 Determination of pH

About 100ml of freshly boiled water at 25℃ was added to a clean, dry flask containing 10g of the sample. Particles were shaken until they were uniformly suspended and lump-free. It was shaken repeatedly for 30 minutes to aid with digestion. After 10 minutes of standing, the supernatant was poured into a 250ml beaker. Then pH was determined using pH paper (AOAC 943.02).

2.4 Statistical Analysis

The data was analysed using R software [R. version 4.1.2 copyright] for statistical computing. Data on the response variables was collected for three replications of the trails and the ANOVA tables was prepared to analyse the data. The critical difference was calculated (*P=.05*), where the F value was significant, and used to identify whether significant differences existed and indicated in the table using superscripts.

$$\text{Critical difference (CD)} = \frac{\sqrt{2} \times MSS(E) \times t\alpha}{r}$$

Where, MSS (E) = Mean Sum of squares of the error
r = number of replications
tα = table t value of the α level of significance

3. RESULTS AND DISCUSSION

3.1 Storage studies of the optimized foxtail millet probiotic dry mix at room temperature (27±1°C)

The storage stability of the optimized foxtail millet probiotic dry mix was assessed over 91 days at ambient temperature (27 ± 1°C). Significant changes were observed in key quality parameters, including probiotic viability, water activity (a_w), moisture content, alcoholic acidity, and pH, highlighting the dynamic impact of storage conditions on product stability.

The viability of probiotics exhibited a gradual but statistically significant decline, decreasing from 11.04 $\log_{10}$CFU/g on day 0 to 8.88 $\log_{10}$CFU/g by day 91. The critical difference (CD) for viability was 0.074 at a 5% significance level, underscoring the measurable reduction in probiotic counts over time. These findings align with studies by Borges *et al.* (2016), who reported a reduction in *Lactiplantibacillus plantarum* viability from 8.9 to 7.7 $\log_{10}$CFU/g in apple powder stored at room

temperature for 90 days. The observed decline can be attributed to environmental stressors such as nutrient depletion and fluctuating temperature and humidity levels.

No fungal or bacterial growth was detected throughout the storage period, consistent with findings by Yadagouda and Ravindra (2022), who observed no microbial growth in foxtail millet composite mixes stored under ambient conditions, and Narayanan and Ravikumar (2017), who reported similar stability in bio-fortified pearl millet flour. The inherent antimicrobial properties of the ingredients, combined with low water activity, likely contributed to this microbial stability.

Water activity (a_w) increased significantly from 0.503 on day 0 to 0.699 by day 91 (CD: 0.010). This trend is consistent with Sharma and Riar (2020), who observed a rise in a_w from 0.40 to 0.71 in millet-based cookies stored for 180 days at room temperature. The hygroscopic nature of millet-based products likely accounts for this increase, even in sealed packaging. Similarly, moisture content increased from 5.37% to 7.68% (CD: 0.073), mirroring the findings of Narayanan and Ravikumar (2017), who documented a rise in moisture content from 6.11% to 9.40% in pearl millet flour stored at 27.5°C for 60 days. This increase can be attributed to the hygroscopic properties of millet flour and potential condensation within packaging due to temperature fluctuations.

Alcoholic acidity rose significantly, from 0.13% on day 0 to 1.78% by day 91 (CD: 0.127). This increase reflects enzymatic and oxidative degradation processes, consistent with Shobha *et al.* (2023), who reported a rise in alcoholic acidity from 0.08% to 1.41% in white finger millet flour stored under ambient conditions. Lipase-mediated fat hydrolysis and protease activity, exacerbated by residual moisture, are likely contributors to this increase.

The pH remained stable at 6.5 until day 49 but decreased significantly to 6.0 by day 56, a level maintained through day 91 (CD: 0.214). This trend is consistent with Goyal *et al.* (2017), who observed a pH reduction in pearl millet flour due to increased moisture, which promotes enzymatic hydrolysis of fats and proteins, releasing free fatty acids and amino acids that lower pH.

According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), a daily intake of at least one billion CFU is required to achieve probiotic health benefits (Hill *et al.*, 2014). The probiotic millet dry mix maintained a viable cell count exceeding this threshold, retaining 9.02 $\log_{10}$CFU/g up to day 84 of storage. These results highlight the product's ability to deliver therapeutic probiotic benefits for a significant portion of its shelf life under ambient conditions, offering a promising functional food solution.

Table 1. Storage studies of the optimized foxtail millet probiotic dry mix at room temperature (27±1°C)

Storage day	Viability	Spores	Yeast & mold	Water activity	Moisture content	Alcoholic acidity	pH
	$\log_{10}$CFU/g				(%)		
0	11.04^{a}	Nil	Nil	0.503^{m}	5.37^{l}	0.13^{j}	6.5^{a}
7	10.87^{b}			0.524^{lm}	5.58^{k}	0.17^{ij}	
14	10.73^{c}			0.539^{kl}	5.75^{j}	0.19^{ij}	
21	10.59^{d}			0.554^{jk}	5.92^{i}	0.25^{ij}	
28	10.31^{e}			0.569^{ij}	6.12^{h}	0.38^{hi}	
35	10.19^{f}			0.584^{hi}	6.34^{g}	0.56^{gh}	
42	10.03^{g}			0.599^{gh}	6.56^{f}	0.78^{fg}	
49	9.81^{h}			0.614^{fg}	6.72^{e}	0.84^{f}	

56	9.63^{i}			0.629^{ef}	6.91^{d}	0.97^{ef}	
63	9.43^{j}			0.643^{de}	7.03^{d}	1.13^{de}	
70	9.31^{k}			0.658^{cd}	7.22^{c}	1.26^{cd}	
77	9.17^{l}			0.674^{bc}	7.34^{c}	1.39^{bc}	6.0^{b}
84	9.02^{m}			0.689^{ab}	7.55^{b}	1.51^{b}	
91	8.88^{n}			0.699^{a}	7.68^{a}	1.78^{a}	
CD(*P=.05*)	0.074	-	-	0.010	0.073	0.127	0.214

Note: CD = Critical Difference and all the values are average of three trials (n=3) and same superscript indicate non-significance while different, indicate statistically significant difference at *P=0.05*

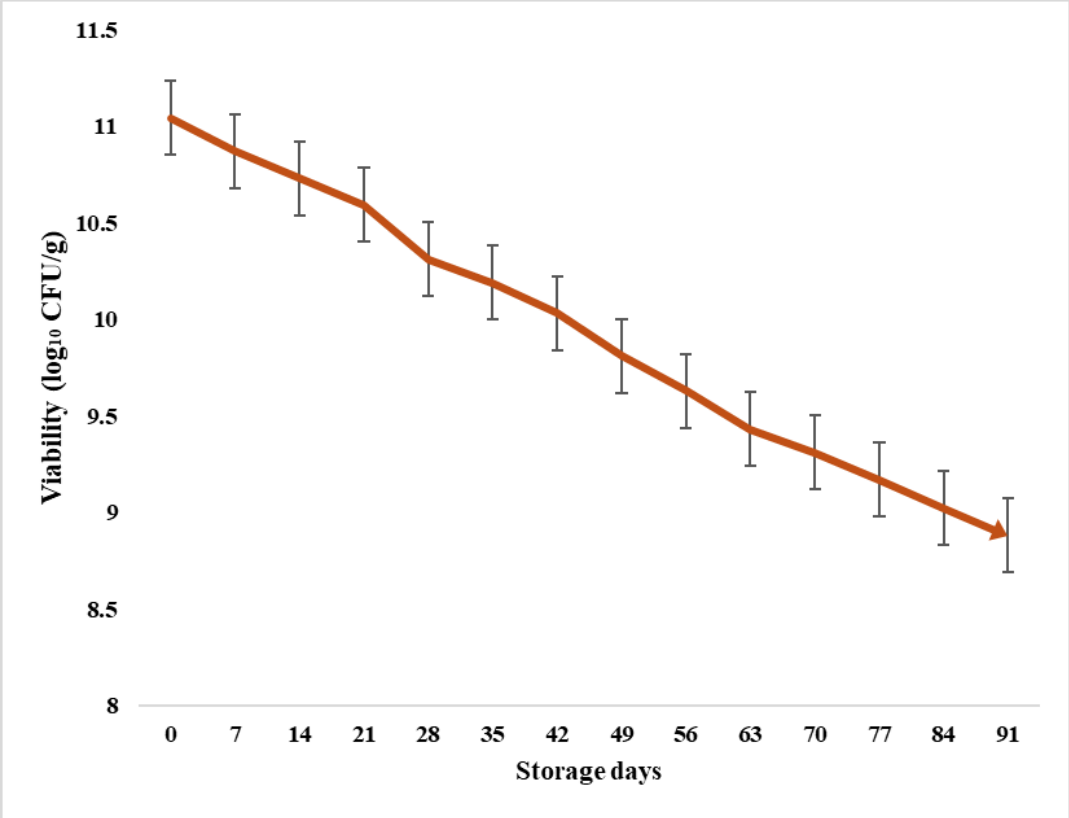

Fig. 1. Viability of probiotics during storage study at room temperature (27±1°C)

3.2 Storage studies of the optimized foxtail millet probiotic dry mix at refrigeration temperature (7±1°C)

The storage study of the optimized foxtail millet probiotic dry mix revealed significant alterations in key quality parameters, including probiotic viability, water activity, moisture content, alcoholic acidity, and pH over 112 days at refrigeration temperature (7 ± 1°C).

The initial viability of the probiotic millet dry mix was recorded at 11.04 $\log_{10}$CFU/g, which declined progressively throughout the storage period. A slight decrease to 10.99 $\log_{10}$CFU/g was observed by day 7. By day 28, the viability showed a significant reduction to 10.64 $\log_{10}$CFU/g, followed by a steady decline to 9.08 $\log_{10}$CFU/g by day 105 and 8.81 $\log_{10}$CFU/g

on day 112. The critical difference (CD) for viability was 0.187 at the 5% significance level, indicating statistically significant reductions over time.

This decline aligns with findings by Bagad *et al.* (2017), who reported a similar trend in freeze-dried *Pediococcus pentosaceus* GS4 cultures stored at 4°C, where viability dropped from 9.0 $\log_{10}$ CFU/ml to below 5.0 $\log_{10}$ CFU/ml. Stability enhancers, such as sorbitol and ascorbic acid, were noted to mitigate viability loss in such systems. Similarly, the viability trends observed in the current study parallel those of freeze-dried probiotic cultures, such as *Lactiplantibacillus plantarum* (Borges *et al.,* 2016), highlighting the gradual yet significant decline over extended refrigerated storage.

No spore formation or yeast and mold counts were detected throughout the storage period, consistent with the observations of Narayanan and Ravikumar (2017) in biofortified pearl millet flour stored at refrigeration temperature (5°C) in high-density polyethylene (HDPE) packaging. The stability in microbial counts is attributed to low water activity and the antimicrobial properties inherent to millet-based formulations.

The water activity of the millet dry mix increased slightly but significantly over the 112 days, rising from an initial value of 0.503 to 0.532. The CD for water activity was 0.002 at the 5% significance level. Comparable studies on milk powders stored at refrigeration temperatures (6 ± 1°C) for 180 days demonstrated minor changes in a_w, such as an increase from 0.299 to 0.315 in skim milk powder and 0.274 to 0.278 in whole milk powder (Ryabova *et al*., 2023). These findings reinforce the importance of low a_w in preserving probiotic stability, as minimal moisture interaction with the environment supports prolonged microbial viability.

Moisture content exhibited a gradual upward trend, increasing from 5.37% on day 0 to 5.73% on day 112, with a CD of 0.028 at the 5% significance level. This increase aligns with observations by Narayanan and Ravikumar (2017), who reported moisture content rising from 6.11% to 9.12% in biofortified pearl millet stored at 5°C for 60 days. The inherent hygroscopic nature of millet flour and the potential for moisture absorption within sealed packaging contribute to these changes.

The alcoholic acidity of the probiotic millet dry mix increased from 0.13% on day 0 to 0.17% by day 112, with a CD of 0.005, indicating statistically significant changes. In contrast, the pH remained stable at 6.5 throughout the storage period, demonstrating non-significant variations. These findings underscore the importance of controlled storage conditions to maintain product quality.

The International Scientific Association for Probiotics and Prebiotics (ISAPP) recommends a minimum daily intake of 1 billion CFU for therapeutic probiotic benefits (Hill *et al*., 2014). The optimized probiotic millet dry mix maintained a viable cell count above this threshold, recording 9.08 $\log_{10}$CFU/g up to day 105 of refrigerated storage. This highlights the formulation's ability to retain probiotic efficacy for over three months under refrigeration.

Table 2. Storage studies of the optimized foxtail millet probiotic dry mix at refrigeration temperature (7±1°C)

Storage day	Viability	Spores	Yeast & mold	Water activity	Moisture content	Alcoholic acidity	pH
	$\log_{10}$CFU/g				(%)		
0	11.04^{a}	Nil	Nil	0.503^{k}	5.37^{k}	0.13^{d}	6.5^{a}
7	10.99^{a}			0.504^{k}	5.37^{k}	0.13^{d}	
14	10.86^{ab}			0.506^{jk}	5.39^{jk}	0.13^{d}	
21	10.78^{ab}			0.509^{ij}	5.42^{ijk}	0.13^{d}	
28	10.64^{bc}			0.509^{ij}	5.42^{ijk}	0.14^{cd}	
35	10.59^{bc}			0.510^{hij}	5.44^{ij}	0.14^{cd}	
42	10.43^{cd}			0.510^{hij}	5.45^{hi}	0.15^{bc}	
49	10.35^{cde}			0.513^{ghi}	5.47^{hi}	0.15^{bc}	

56	10.22^{de}			0.514^{fgh}	5.50^{gh}	0.15^{bc}	
63	10.18^{de}			0.514^{fgh}	5.54^{fg}	0.15^{bc}	
70	10.05^{ef}			0.517^{efg}	5.56^{ef}	0.15^{bc}	
77	9.82^{fg}			0.518^{ef}	5.58^{def}	0.16^{ab}	
84	9.64^{gh}			0.521^{de}	5.60^{de}	0.16^{ab}	
91	9.42^{hi}			0.523^{cd}	5.63^{cd}	0.16^{ab}	
98	9.25^{i}			0.526^{bc}	5.67^{bc}	0.16^{ab}	
105	9.08^{ij}			0.529^{ab}	5.71^{ab}	0.16^{ab}	
112	8.81^{j}			0.532^{a}	5.73^{a}	0.17^{a}	
CD (*P=.05*)	0.187	-	-	0.002	0.028	0.005	-

Note: CD = Critical Difference and all the values are average of three trials (n=3) and same superscript indicate non-significance while different, indicate statistically significant difference at *P=.05*

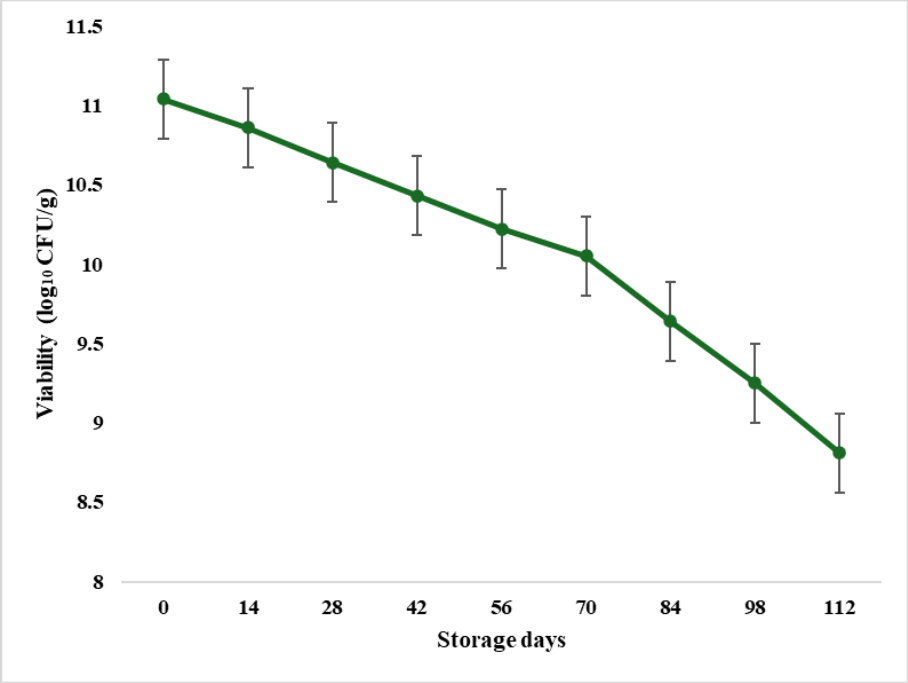

Fig. 2. Viability of probiotics during storage study at refrigeration temperature (7±1°C)

4. CONCLUSION

The storage study of foxtail millet probiotic powder enriched with high protein and fibre demonstrated its potential as a stable functional food product under both refrigeration (7 ± 1°C) and ambient (27 ± 1°C) storage conditions. Probiotic viability remained above the critical threshold of 1 billion CFU/g for up to 105 days under refrigeration and 84 days under ambient conditions, ensuring the product's ability to deliver therapeutic benefits. Changes in water activity, moisture content,

alcoholic acidity, and pH were statistically significant across storage durations, reflecting dynamic interactions between the product's physicochemical properties and environmental factors.

Low water activity and the inherent antimicrobial properties of the ingredients effectively inhibited fungal and bacterial contamination throughout the storage period, enhancing the product's microbiological stability. The gradual increase in water activity and moisture content highlights the need for optimized packaging solutions to mitigate moisture absorption, particularly under ambient conditions.

The findings underscore the feasibility of developing a shelf-stable, high-protein, and high-fibre probiotic millet powder that retains its functional and nutritional attributes during extended storage. These results also pave the way for further studies on advanced packaging technologies and storage condition optimizations to extend the shelf life of millet-based functional foods, thereby expanding their applicability in the growing health-conscious consumer market.

ABBREVIATIONS

AOAC: American Association of Agricultural Chemists

FSSAI: Food Safety and Standards Authority of India

MEA: Malt Extract Agar

MRS: de Man Rogosa Sharpe Agar

NA: Nutrient Agar

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