Characterization and standardization of a millet-based probiotic beverage via physicochemical and microbial analysis

ABSTRACT

Aims: This study aimed to explore the potential of millet, a gluten-free grain, in developing a probiotic-rich fermented beverage. The focus was on assessing millet's physicochemical properties for beverage production, optimizing a standardized formulation for sensory appeal and nutritional quality, and evaluating the beverage's shelf-life parameters**.**
Study design: Experimental study design
Place and Duration of Study: Department of Food & Nutrition, School for Home Science, Babasaheb Bhimrao Ambedkar (a central) University, Lucknow, Uttar Pradesh, during the period of September 2023 to April 2024
Methodology: Physicochemical properties of millet were analyzed to determine its suitability for fermentation. Lactic acid bacteria and Bacillus coagulans were used as the probiotic culture, and fermentation was conducted at 32°C for 12 hours. Parameters such as acidity, pH, Total Soluble Solids (TSS), protein, fat, ash, crude fiber, phytochemical content, ~~and probiotic viability~~ were measured to assess the beverage's quality and shelf-life.
Results: The probiotic millet beverage demonstrated acceptable sensory acceptance and shelf-life when stored at 6°C for 14 days. After 7 days of storage, viable probiotic cell counts were recorded at 2.910 CFU/mL. These results suggest the feasibility of using millet for probiotic beverage production, providing a nutritious option that leverages the health benefits of this gluten- grain.
Conclusion: This research underscores the suitability of millet for developing probiotic-rich beverages, offering a functional and nutritious alternative that addresses both sensory and nutritional considerations. The study also highlights the potential of millet-based beverages in addressing nutritional deficiencies and promoting gut health, especially in communities with limited access to diverse food sources.

Keywords: Beverage, Fermentation, Lactic acid bacteria, Nutrition, Probiotic , Shelf- life

1. INTRODUCTION

Consumer preferences have shifted significantly in favour of healthier and more nutrient-dense food and beverage options in recent years. Growing interest in functional foods—foods that provide health advantages beyond basic nutrition—and an improving understanding of the relationship between diet and health are the main drivers of this movement.[1] Among the various categories of functional foods, probiotic beverages have emerged as a popular choice due to their potential to promote gut health, boost immunity, and improve overall well-being.Probiotics are live microorganisms that, when consumed in adequate amounts, confer health benefits to the host. They are commonly found in fermented foods and beverages such as yogurt, kefir, and kombucha. Probiotic beverages, in particular, have gained traction among health-conscious consumers looking for convenient and tasty ways to incorporate probiotics into their daily diet.

Millets, a group of small-seeded grasses cultivated and consumed in many parts of the world, offer a unique opportunity for the development of probiotic-rich beverages.[2] Millets are known for their nutritional richness, gluten-free nature, and resilience to adverse environmental conditions. They are rich sources of essential nutrients such as vitamins, minerals, and dietary fibre, making them an ideal substrate for the production of nutritious and functional food products.The combination of millets and probiotics in the form of millet-based probiotic drinks represents an innovative approach to delivering health-promoting benefits to consumers. Millets provide a natural and sustainable source of nutrients and prebiotic fibres that can support probiotic growth and enhance their viability and functionality in the beverage. By harnessing the nutritional and functional properties of millets, along with the health-promoting properties of probiotics, millet-based probiotic drinks have the potential to offer a wide range of health benefits to consumers.[3] Despite the growing interest in probiotic beverages and the nutritional potential of millets, there is currently limited research on the development and standardization of millet-based probiotic drinks. This gap in knowledge presents an opportunity for scientific investigation and innovation in the field of

functional foods. The aim of this research is to address this gap by comprehensively evaluating the physicochemical and microbiological properties of millet-based probiotic drinks and developing a standardized formulation that meets consumer preferences, regulatory requirements, and industry standards. Through rigorous scientific inquiry and optimization of processing parameters, this research seeks to establish a robust framework for the production of millet-based probiotic drinks that ensures product safety, efficacy, and consistency. By providing consumers with a nutritious and sustainable alternative to traditional probiotic products, millet-based probiotic drinks have the potential to make a meaningful impact on public health and well-being.

2. MATERIAL AND METHODS

The current study was conducted at Babasaheb Bhimrao Ambedkar University (BBAU), Lucknow in the Food Analysis Laboratory and the Laboratory of Microbiology, Department of Food and Nutrition.

2.1. *Selection of Millet Varieties:*

Finger millet (Eleusine coracana) was taken from the local shop for the probiotic drink.

2.2. *Preparation of Ragi Flour:*

In the process of preparing ragi flour, the first step involves thoroughly cleaning the raw ragi (finger millet) grains to remove any impurities, debris, or foreign matter. This cleaning process ensures that the grains are free from contaminants and ready for further processing. Once cleaned, the grains are then milled to obtain fine flour. Milling is a mechanical process that involves grinding or crushing the grains to break them down into smaller particles. This results in a smooth and uniform texture. After milling, the flour undergo additional processing steps such as sieving to remove any coarse particles and ensure consistency.[4]

2.3. *Preparation of Starter Culture:*

A commercially available probiotic starter culture containing strains of Lactobacillus spp. was used for fermentation. The starter culture was prepared according to the manufacturer's instructions.

2.4. *Formulation of Millet-Based Probiotic Drink:*

In a sterile container, ragi flour was mixed with distilled water to form a smooth paste. The ragi paste mixture was autoclaved along with water for 15-20 minutes at 121°C to ensure sterilization. After autoclaving, the mixture was allowed to cool to room temperature. The starter culture was added to the cooled mixture at a predetermined concentration

Ragi flour was mixed with distilled water to form a smooth paste.

↓

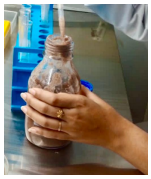
The ragi paste was autoclaved along with water at 121°C for 20 minutes.

↓

The autoclaved mixture was allowed to cool to room temperature.

↓

The prepared starter culture was added to the cooled mixture.



↓

The mixture was put in the incubator for fermentation.

Chart 1 : Formulation of the probiotic drink

2.5. Fermentation Process:

The mixture was transferred to sterile containers or fermentation vessels. The containers were sealed to prevent contamination and placed in an incubator set to 32°C. Fermentation was allowed to proceed for 8-12 hours under controlled conditions.

2.6. Physicochemical Analysis:

Using conventional techniques, a nutritional composition analysis was carried out to ascertain the probiotic drink made from millet's proximate composition (moisture, ash, protein, fiber, fat and carbohydrate content).

2.6.1 Sample Preparation:

Ragi probiotic drink samples were collected and stored under refrigeration prior to analysis. Samples were homogenized to ensure uniformity before analysis.

2.6.2 PH Measurement:

A calibrated pH meter was used to determine the pH of every sample. After the electrode was stabilized, the sample was submerged in it, and the pH value was noted.[6]

2.6.3 Total Solids Determination:

Total solids content was determined by weighing a known volume of the sample before and after drying at 105°C until a constant weight was obtained.[7]

$$\text{Total solids}(\%) = \frac{\text{(Initial Mass - Final Mass)}}{\text{Initial Mass}} \times 100\%$$

Where:

- Initial Mass is the mass of the sample before drying
- Final Mass is the mass of the sample after drying to a constant weight at 105°C

2.6.4 Total Acidity Analysis: Total acidity was determined by titrating the sample with standardized sodium hydroxide (NaOH) solution to the phenolphthalein endpoint.[8]

$$\text{Total Acidity}(g/L) = \frac{(V_{NaOH} \times N_{NaOH} \times M_{NaOH})}{V_{sample}}$$

Where:

- V_{NaOH} is the volume of sodium hydroxide solution used in titration (in liters).
- N_{NaOH} is the normality of the sodium hydroxide solution (in mol/L).
- M_{NaOH} is the molar mass of sodium hydroxide (in g/mol).
- V_{sample} is the volume of the sample used for titration (in liters).

2.6.5 Brix measurement:

Brix content was determined using a refractometer, which measures the refractive index of the sample. A known volume of the sample was placed on the refractometer's prism, and the refractive index was read directly from the instrument's scale. Brix values represent the percentage of soluble solids in the sample, primarily sugars, and are commonly used in the food and beverage industry to assess product quality and sweetness levels.[9]

2.6.6 Protein Content Analysis:

The Kjeldahl method, which entails numerous procedures including digestion, distillation, and titration, was used to determine the protein levels. The sample was processed by combining a combination with strong sulfuric acid. Distillation was carried out with the addition of sodium hydroxide (NaOH) after dilution. After that, the distillate was collected and placed in a conical flask with boric acid and an indicator. The mixture was then stirred until a color shift took place. The distillate was then titrated against standard hydrochloric acid to ascertain the protein level.[10]

$$\text{Percentage of protein} = \frac{(c - b) \times 14 \times d \times 6.25 \times 100}{a \times 100}$$

Where:

a = sample weight (gm)
b = volume of NaOH necessary for titration for sample
c = volume of NaOH required for titration for blank
d = normality of NaOH used for titration. The conversion factor is 6.25; and the atomic weight of nitrogen is 14.

2.6.7 Fat Content Determination:

Through the use of a solvent to extract fat from the sample, evaporation, and weighting, the Soxhlet extraction method was used to determine the amount of fat present.Crude fat was examined using the soxhlet extraction method. In pre-weighed thimbles, five-gram sample was taken. Petroleum ether was used during the six-hour extraction process.[11]

$$\text{Weight of Fat} = \frac{(W2-W1)}{\text{Weight of the Sample}} \times 100$$

Where:

W1 = weigh of beaker

W2 = weigh of beaker with fat

2.6.8 Ash Content Measurement:

Ash was measured by accurately weighing five to ten grams of the material into a crucible that had been previously weighed. To make sure that every item burned except the food's minerals, the sample was torched at 600°C.[12]

Percentage of Ash = (Mass of Ash / Mass of Sample)

2.6.9 Crude Fibre Analysis:

Fibre plus was used to calculate the amount of crude fiber. Sodium hydroxide (0.313N) and sulfuric acid (0.255N) solvents were used for basic and acid digestion, respectively. Following boiling, the extract was put in a muffle furnace for 30 minutes to remove any carbonaceous elements , and the amount of weight lost was determined to be crude fiber.[13]

$$\text{Percentage of fibre} = \frac{\text{Mass of Sample}}{\text{Mass of Fibre}} \times 100$$

2.6.10 Phytochemical Analysis:

Phytochemical analysis of the ragi probiotic drink was conducted using specific reagents to identify bioactive compounds presen his method offers insights into the drink's potential health benefits and nutritional composition, facilitating its characterization for therapeutic applications.[14]

Table no.1: The details of phytochemical analysis along with methodology

Phytochemical	Test Method	Procedure
Phenolic Compounds	FeCl3 test	2 ml of distilled water and 3–5 drops of ferric chloride solution added to 1 ml of sample
Flavonoids	NaOH test	A few drops of 2N NaOH solution added to 1 ml of the sample
Tannins	Braemer's test	20% alcoholic ferric chloride added to two ml of sample

2.7 Microbiological Analysis

Microbial enumeration was conducted to determine the total viable count (TVC) of bacteria in the sample using agar plate count methods.The number of live bacterial cells in the ragi probiotic drink was determined using the plate count method. The sample was prepare ,in the sample preparation stage, the culture was progressively diluted in phosphate buffered saline (PBS) to achieve dilutions suitable for subsequent plating.[15] Subsequently, for the plate count method, triplicate dilutions were spread onto nutrient agar plates using the spread plate technique, with each plate receiving 100 µl of the diluted suspension. These plates were then incubated aerobically at 37°C for approximately 3 days to allow microbial growth. After the incubation period, the plates were examined, and the colonies were counted, with the results reported as Colony Forming Units per milliliter (CFU/mL). Additionally, to evaluate the vitality of bacterial cells in the juice, a spread plate technique was employed. Serial dilutions of samples were prepared every 24 hours to ensure countable colonies, and these diluted samples were evenly spread onto nutrient agar plates using sterile spreaders. The nutrient agar plates were subsequently incubated overnight at 37°C to promote microbial growth.[16]

Viable cell count = (number of colonies)/ (dilution x amount plated)

Chart 2 : Stepwise procedure of Microbial analysis

2.8. Sensory Evaluation:

Trained sensory panellists evaluated the sensory attributes of the millet-based probiotic drink, including appearance, aroma, taste, texture, and overall acceptability, using standardized sensory evaluation methods that is the hedonic rating test.[17]

3. RESULTS AND DISCUSSION

3.1 Physicochemical Analysis:

The sample underwent fermentation for a duration of 8-12 hours to assess its chemical properties and probiotic viability. During this short fermentation period, notable changes were observed in the beverage's pH and acidity levels. Initially, the pH of the drink increased gradually, attributed to its inherent buffering capacity and the metabolic activity of select Lactobacillus strains. This metabolic activity led to the production of lactic acid, contributing to the gradual rise in acidity. Subsequently, the pH gradually declined, indicating a reduction in the beverage's buffering capacity as fermentation progressed. The beverage exhibited its highest acidity of 0.70 percent, indicative of active probiotic metabolism. Despite the short fermentation duration, the total sugar content remained relatively stable, ensuring the retention of essential nutrients such as protein, fat, and minerals within acceptable ranges. Overall, fermentation for 8-12 hours resulted in a well-rounded and nutritionally rich ragi probiotic drink, showcasing its potential as a functional beverage with enhanced health benefits.

Table no.2: Physiochemical analysis

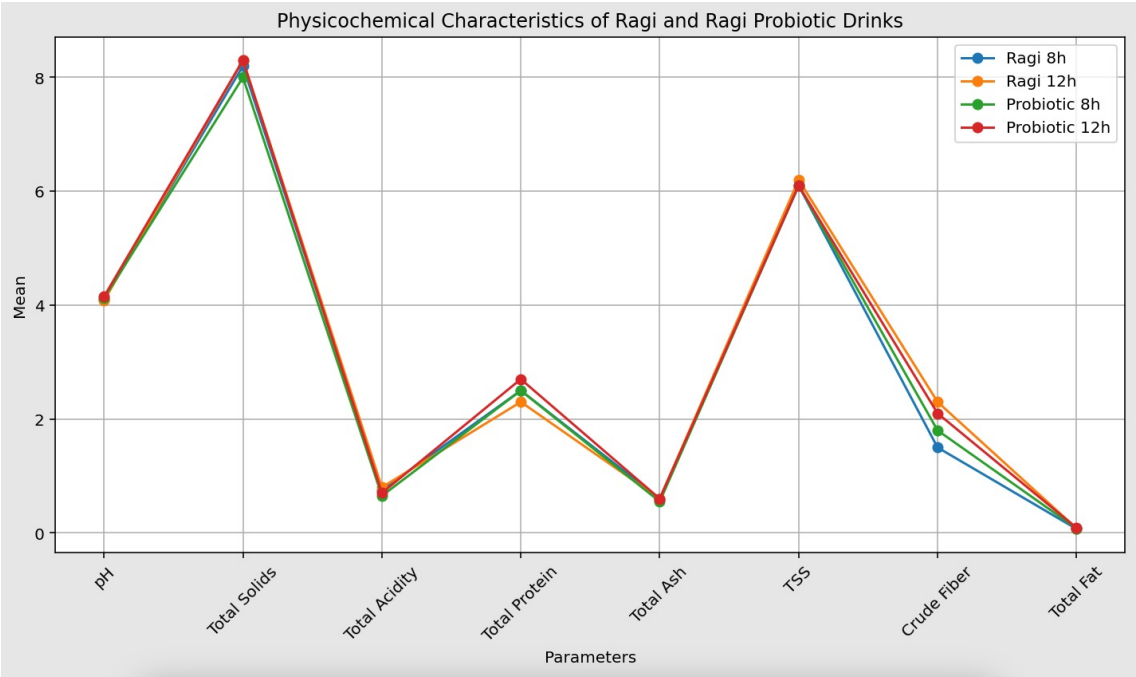
Parameter	Ragi Drink (8 hours)	Ragi Drink (12 hours)	Ragi Probiotic Drink (8 hours)	Ragi Probiotic Drink (12 hours)
pH	4.10 ± 0.05	4.08 ± 0.05	4.12 ± 0.05	4.15 ± 0.05
Total Solids (%)	8.2 ± 0.2	8.3 ± 0.2	8.0 ± 0.2	8.3 ± 0.2
Total Acidity (%)	0.75 ± 0.03	0.80 ± 0.03	0.65 ± 0.03	0.70 ± 0.03
Total Protein (%)	2.5 ± 0.2	2.3 ± 0.2	2.5 ± 0.2	2.7 ± 0.2
Total Ash (%)	0.6 ± 0.03	0.6 ± 0.03	0.55 ± 0.03	0.60 ± 0.03
TSS (Brix%)	6.1 ± 0.5	6.2 ± 0.5	6.1 ± 0.5	6.1 ± 0.5
Crude Fiber (%)	1.5 ± 0.2	2.3 ± 0.3	1.8 ± 0.4	2.1 ± 0.2
Total fat (%)	0.07% ± 0.01%	0.08% ± 0.01%	0.08% ± 0.01%	0.09% ± 0.01%

The table presents the physicochemical characteristics of ragi drink fermented for 8 and 12 hours, as well as ragi probiotic drink fermented for the same durations. pH values of the drinks ranged from 4.08 to 4.12, with slight variations observed between fermentation durations.

Total solids content ranged from 8.0% to 8.3%, indicating consistency in the overall composition of the drinks. Total acidity levels showed a similar trend, with values ranging from 0.65% to 0.70%.

Protein content varied from 2.5% to 2.7%, with higher values observed in the 12-hour fermented drinks. Fat content remained relatively consistent across all samples, ranging from 0.7% to 0.9 %. The ash content was also consistent, with values ranging from 0.55% to 0.6%.

Total phenolic and flavonoid content, remained stable across all samples, indicating the retention of bioactive compounds during fermentation. Overall, the results demonstrate the feasibility of producing ragi-based probiotic drinks with consistent physicochemical characteristics and bioactive compounds, which are essential for promoting health benefits.



Graph 1: Graphical representation of Physiochemical analysis

3.2 Phytochemical Analysis

Table 3: Phytochemical Analysis

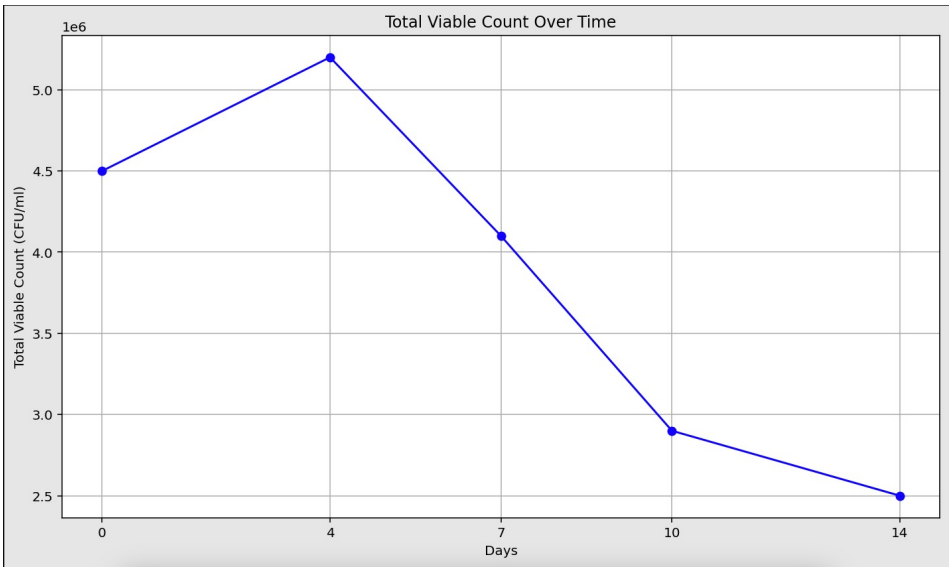
Phytochemical	Color Indication	Presence
Flavonoids	Yellow	Yes
Phenolic Compounds	Blue-Green	Yes
Tannins	Dark blue	Yes

3.3 Microbiological analysis

The total viable cell count of the advancement beverage sample was examined at 14 days (see Table 4). As the storage period progressed, there was a notable increase in the total viable count (TVC). The microbial growth pattern showed a gradual and steady expansion of probiotic bacteria from 0 to 7 days of storage, followed by a significant decline. This observation aligns with previous research indicating that the TVC of *Lactobacillus reuteri* and *Bifidobacterium bifidum* in whey-based probiotic beverages decreased after 30 days of storage at 41°C. However, the prepared beverage maintained a viable probiotic count within a safe range, suggesting it could be considered a functional dose for human consumption for up to 14 days at 6°C.[18]

Table no.4 : Total viable count (cfu/ml)

DAY	DILUTION	Total viable count (CFU/ml)
0	10^-3	4.5 x 10^6
4	10^-3	5.2 x 10^6
7	10^-3	4.1 x 10^6
10	10^-3	2.9 x 10^6
14	10^-3	2.5 x 10^6



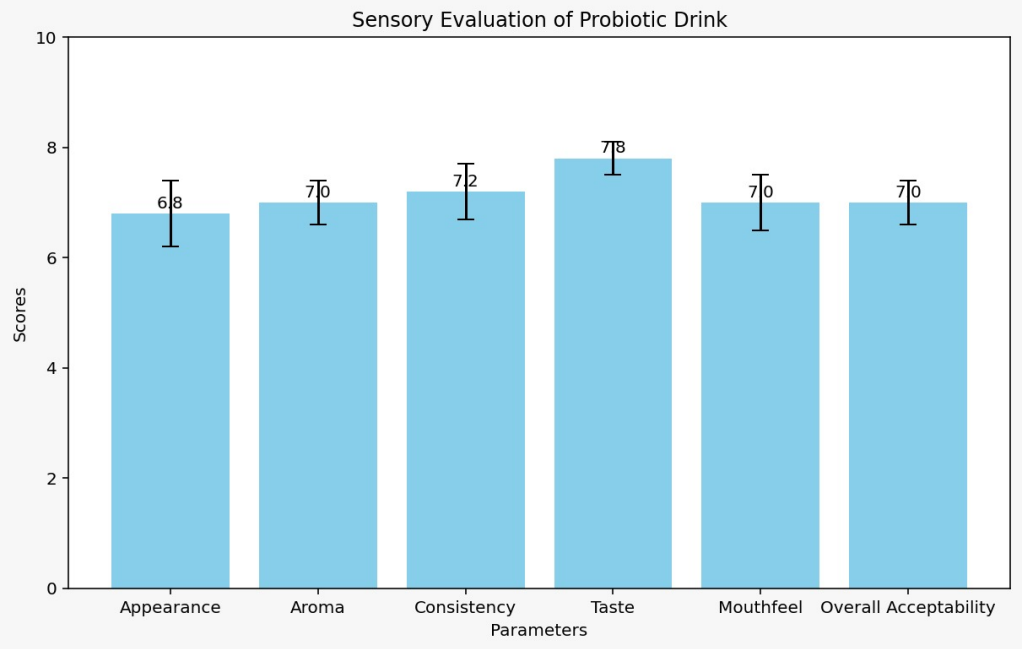
Graph 2: Graphical representation of TVC over time

3.4 Sensory Evaluation

Acceptance evaluations were carried out using a 9-point hedonic scale, with ratings ranging from 1 for extreme dislike to 9 for extreme liking .[19] The sensory evaluation involved 40 semi trained participants, comprising 45% males and 55% females, Participants were randomly selected from both within the University Samples (50 ml) were served chilled in transparent plastic cups. All sensory evaluations were conducted in individual booths. The sensory evaluation results indicated overall positive acceptance of the millet-based probiotic drink among the consumers. Panellists described the drink as having a pleasant aroma, mild flavour profile, and smooth texture, with no off-flavours or unpleasant aftertaste.Appearance and colour were rated favourably, contributing to the visual appeal of the product.[20]

Table 5: Sensory evaluation

Sample	Appearance	Aroma	Taste	Consistency	Acceptance	Mouthfeel
Ragi probiotic drink	6.8 ± 0.6	7.0 ± 0.4	7.8 ± 0.3	7.2 ± 0.5	7.0 ± 0.4	7.0 ± 0.4



Graph no.3: Graphical representation of sensory analysis

4. CONCLUSION

In conclusion, the successful development and standardization of a millet-based probiotic drink have been achieved through comprehensive physicochemical and microbiological analysis. The results demonstrate the potential of millets as a versatile substrate for producing nutritious and functional beverages with probiotic benefits. Physicochemical analysis revealed favorable nutritional composition, pH, titratable acidity, ash, crude fiber and phytochemical indicating the drink's suitability for probiotic fermentation and consumer acceptability. Microbiological analysis confirmed the presence and viability of probiotic strains, ~~such as Lactobacillus spp. and Bifidobacterium spp.~~ highlighting its potential to promote gut health. Sensory evaluation results indicated positive consumer acceptance, with favorable ratings for aroma, flavor, texture, and appearance, suggesting broad appeal among consumers seeking nutritious and palatable probiotic beverages. Overall, the development of the millet-based probiotic drink represents a significant contribution to functional foods and nutrition, offering a sustainable and health-promoting option for improving gut health and overall well-being. Future research may focus on optimizing formulation and processing parameters to enhance nutritional profile, sensory attributes, and shelf stability, along with conducting clinical studies to validate its efficacy in supporting gut health and wellness.

REFERENCES

1. Roberfroid, M. B. (2000). Concepts and strategy of functional food science: The European perspective. *American Journal of Clinical Nutrition, 71*(6), 1660S-1664S.

2. Shobana, S., Krishnaswamy, K., Sudha, V., Malleshi, N. G., Anjana, R. M., Palaniappan, L., & Mohan, V. (2013).

3. Sharma, S., & Sharma, N. (2021). Preparation of probiotic enriched functional beverage of Kodo millet (*Paspalum scrobiculatum*): A nutritionally enriched absolute new product for commercialization. *Journal of Pharmacognosy and Phytochemistry, 10*(1), 752-758.

4. Desai, A. D., Kulkarni, S. S., Sahoo, A. K., Ranveer, R. C., & Dandge, P. B. (2010). Effect of supplementation of malted ragi flour on the nutritional and sensorial quality characteristics of cake. *Advances in Journal of Food Science and Technology, 2*(1), 67-71.

5. Fasreen, M. M. F., Perera, O. D. A. N., & Weerahewa, H. L. D. (2017). Development of finger millet based probiotic beverage using *Lactobacillus casei*431.

6. Westcott, C. (2012). pH measurements. *Elsevier*.

7. Elechi, J. O. G., Abu, J. O., & Eke, M. O. (2023). The application of blends of bambaranut and millet vegetable milk in the development of plant-based yoghurt analogues: Proximate composition, physiochemical properties, microbial safety and consumer's acceptability. *Food and Health, 9*(1), 43-60.

8. Ratau, M. A. (2018). Chemometrics, physicochemical and sensory characteristics of pearl millet beverage produced with bioburden lactic acid bacteria pure cultures [Doctoral dissertation, Cape Peninsula University of Technology].

9. Deelen, S. M., Ollivett, T. L., Haines, D. M., & Leslie, K. E. (2014). Evaluation of a Brix refractometer to estimate serum immunoglobulin G concentration in neonatal dairy calves. *Journal of dairy science, 97*(6), 3838-3844.

10. Lynch, J. M., & Barbano, D. M. (1999). Kjeldahl nitrogen analysis as a reference method for protein determination in dairy products. *Journal of AOAC International, 82*(6), 1389-1398.

11. Folch, J., Lees, M., & Stanley, G. S. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry, 226*(1), 497-509.

12. Abadi, F. R. (2018). Design of a simple muffle furnace for temperature optimization in ash content analysis. *Jurnal Neutrino: Jurnal Fisika dan Aplikasinya, 10*(2), 30-39.

13.Madhu, C., Krishna, K. M., Reddy, K. R., Lakshmi, P. J., & Kelari, E. K. (2017). Estimation of crude fibre content from natural food stuffs and its laxative activity induced in rats. *Int J Pharma Res Health Sci, 5*(3), 1703-1706.

14.Wadood, A., Ghufran, M., Jamal, S. B., Naeem, M., Khan, A., & Ghaffar, R. (2013). Phytochemical analysis of medicinal plants occurring in the local area of Mardan. *Biochemical and Analytical Biochemistry, 2*(4), 1-4.

15.Thakur, M., & Sharma, R. K. (2017). Development of probiotic pomegranate beverage and its physico-chemical and microbial characterization. *Int. J. Pure App. Biosci, 5*(1), 35-41.

16.Khatoon, N., & Gupta, R. K. (2015). Probiotics beverages of sweet lime and sugarcane juices and its physiochemical, microbiological & shelf-life studies. *Journal of Pharmacognosy and Phytochemistry, 4*(3), 25-34.

17.Dias, M. D. L. L. A., Salgado, S. M., Guerra, N. B., Livera, A. V. S., Andrade, S. A. C., & Ximenes, G. N. D. C. (2013). Phisicochemical, sensory, and microbiological evaluation and development of symbiotic fermented drink. *Food Science and Technology, 33*, 805-811.

18.Skryplonek, K., Dmytrów, I., & Mituniewicz-Małek, A. (2019). Probiotic fermented beverages based on acid whey. *Journal of dairy science, 102*(9), 7773-7780.

19.Gatti, E., Di Virgilio, N., Magli, M., & Predieri, S. (2011). Integrating sensory analysis and hedonic evaluation for apple quality assessment. *Journal of Food Quality, 34*(2), 126-132.

20.Sharif, M. K., Butt, M. S., Sharif, H. R., & Nasir, M. (2017). Sensory evaluation and consumer acceptability. In *Handbook of food science and technology*, 10, 361-386.