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Characterization and Standardization of a Millet-based Probiotic Beverage Via Physicochemical and Microbial Analysis

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Authors' contributions

This work was carried out in collaboration among all authors. Author SP designed the methodology and wrote original draft. Author AN did software analysis, wrote, reviewed and edited the manuscript. Author NS did data validation and supervised the study. All authors read and approved the final manuscript.

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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.

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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 clausii were used as the probiotic culture, and fermentation was conducted at 32°C for 8h and 12h. Parameters such as acidity, pH, Total Soluble Solids (TSS), protein, fat, ash, crude fiber, phytochemical content, and microbial profile was evaluated during 14 day storage period at 6°C and sensorial evaluation was performed at the end of the storage period.
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. 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. MATERIALS 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

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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 in Samridhi Krishi Yantra Udyog to obtain fine flour. Following milling, the flour undergoes additional processing steps, including sieving with a pore size of 1mm, aimed at removing coarse particles and ensuring 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, 30 grams of ragi flour were combined with 300 milliliters of distilled water to form a smooth paste, maintaining the ratio of 1:10 between the ragi flour and water. The resulting 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 then added to the cooled mixture at a predetermined concentration for the preparation of 300ml of ragi probiotic drink [5].

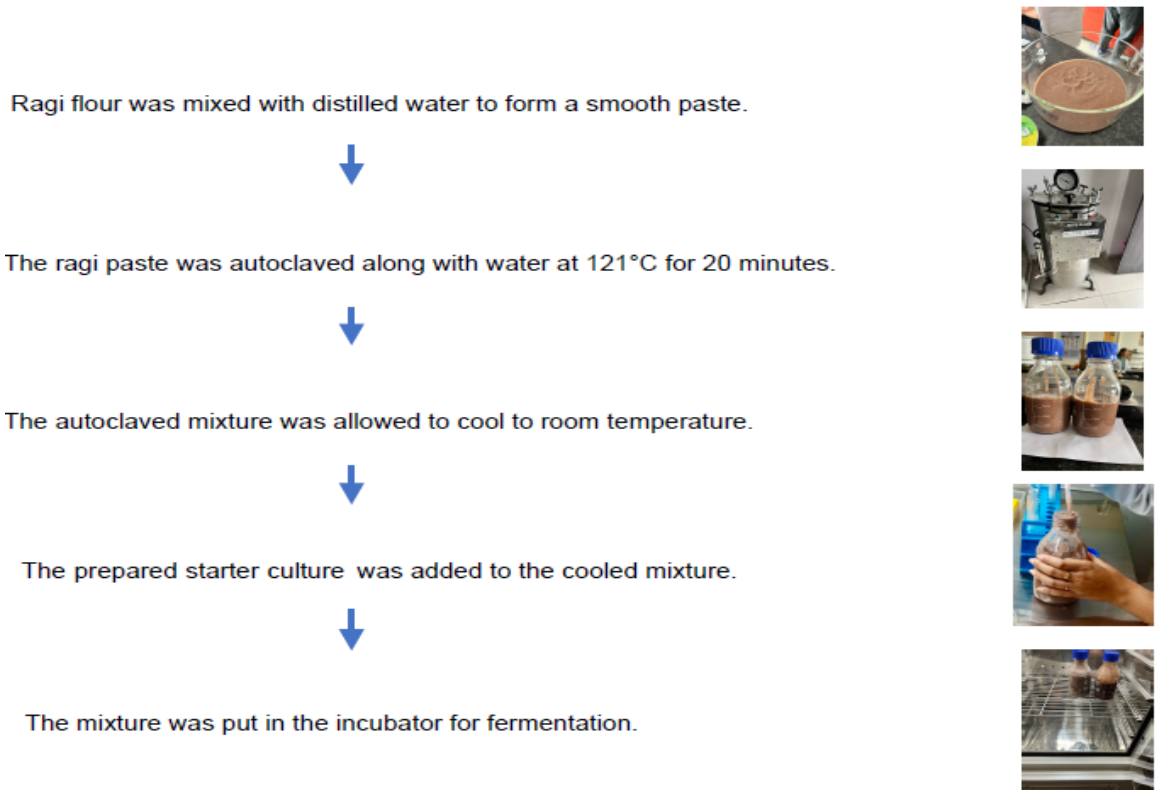


Chart 1. Formulation of the probiotic drink

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, specifically the GEC-P40605V, a table-top digital pH meter manufactured by GLOBAL ENGINEERING CORPORATION for laboratory use, was employed to determine the pH of each sample. Once the electrode stabilized, the sample was submerged in it, and the pH value was measured [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].

Total solids (%) = (Final Mass / Initial Mass) x 100%

Where:

Initial Mass: The mass of the sample before drying, expressed in grams (g).

Final Mass: The mass of the sample after drying to a constant weight at 105°C, also expressed in grams (g).

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].

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

Where:

- VNaOH is the volume of sodium hydroxide solution used in titration (in liters).
- NNaOH is the normality of the sodium hydroxide solution (in mol/L).
- MNaOH is the molar mass of sodium hydroxide (in g/mol).
- Vsample 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].

Percentage of protein = ((c - b) x 14 x d x 6.25 x 100 / a x 100)

Where:

a = sample weight (g);
b = volume of NaOH necessary for titration for sample(mL);
c = volume of NaOH required for titration for blank(mL);

d = normality of NaOH used for titration (moles per liter, M); 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].

Weight of Fat = [(W2-W1)/Weight of the Sample] x 100

Where:

W1 = weight of beaker in grams(g)
W2 = weight of beaker with fat in grams (g)

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].

Percentage of fibre = (Mass of Sample /Mass of Fibre) × 100

2.6.10 Phytochemical Analysis

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

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)

Table 1. The details of phytochemicalanalysis along with methodology

Phytochemical	Test Method	Procedure
Phenolic Compounds	Fecl3 Test	2ml of distilled water and 3-5 drops of ferric chloride solutionadded to 1ml of sample
Flavonoids	NaOH Test	A few drops of 2N NaOH solutionadded to 1ml of the sample
Tanins	Braemers's Test	20% alcoholic ferric chloride added to 2ml of sample

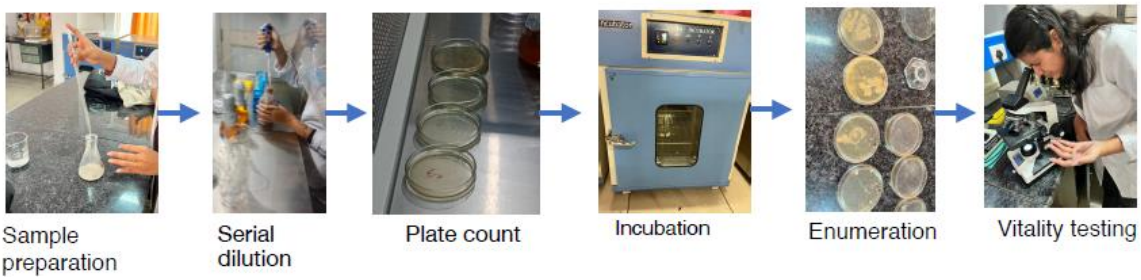


Chart 2. Stepwise procedure of Microbial analysis

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]. Acceptance evaluations were carried out using a 9-point hedonic scale, with ratings ranging from 1 for extreme dislike to 9 for extreme liking [18]. 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.

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.Similar result was also observed in whey based pineapple probiotic drink [19].

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%.

Table 2. Physiochemical analysis

Parameter	Ragi Drink (8 hours)	Ragi Drink (12 hours)	Ragi probiotic drink (8 hours)	Ragi probiotic drink (12 hours)
pH	410±0.05	4.8±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%±0.05%	0.08%±0.01%	0.09%±0.01%

Table 3. Statistically assessed table

Table Analyzed	Data 4				
Data sets analyzed	A-D				
ANOVA summary					
F	0.9881				
P value	0.4126				
P value summary	ns				
Significant diff. among means (P< 0.05)?	No				
R square	0.09573				
Brown-Forsythe test					
F (DFn, DFd)	1.001 (3, 28)				
P value	0.4068				
P value summary	ns				
Are SDs significantly different (P< 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	118.0				
P value	<0.0001				
P value summary	****				
Are SDs significantly different (P< 0.05)?	Yes				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	15384	3	5128	F (3, 28) = 0.9881	P=0.4126
Residual (within columns)	145310	28	5190		
Total	160693	31			
Data summary					
Number of treatments (columns)	4				
Number of values (total)	32				

Table 4. Phytochemical Analysis

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

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% to0.6%.

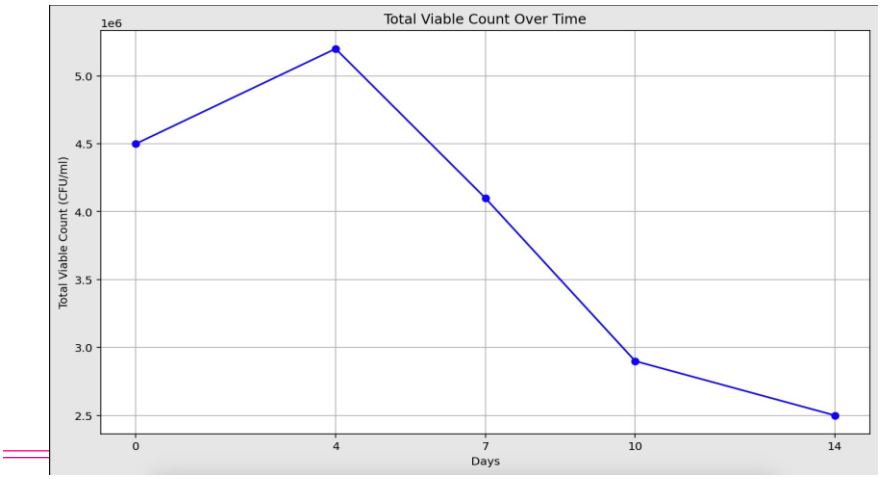
Total phenolic and flavonoid content, remained stable across all samples, indicating the retention of bioactive compounds during fermentation. Overall, the results demonstrate the feasibility of producing ragi-based probiotic drinks with consistent physicochemical characteristics and bioactive compounds,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

3.2 Phytochemical Analysis

The experiment elucidates the presence and visual characteristics of key phytochemicals within the sample. Three prominent phytochemicals are examined: Flavonoids, Phenolic Compounds, and Tannins. Flavonoids are identified by a yellow coloration, Phenolic Compounds by a blue-green hue, and Tannins by a dark blue appearance. The comprehensive analysis confirms the presence of all three phytochemicals in the sample, underscoring their significant contribution to its chemical composition [14].

3.3 Microbiological Analysis

The total viable cell count of the advancement beverage sample which was fermented for 12hrs was examined at 14 days (. 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



Graph 1. Graphical representation of TVC over time

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

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 [20].

3.4 Sensory Evaluation

The sensory evaluation results indicated overall positive acceptance of the millet-based probiotic drink among the consumers. The sample fermented for 12hrs was taken for the sensory evaluation.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 [21-23].

4. CONCLUSION

In conclusion, the successful development and standardization of a millet-based probiotic drink have been accomplished through comprehensive physicochemical and microbiological analysis. The results underscore the potential of millets as a versatile substrate for creating nutritious and functional beverages with probiotic benefits. Physicochemical analysis unveiled a favorable nutritional composition, pH, titratable acidity, ash, crude fiber, and phytochemical content, indicating the drink's suitability for probiotic fermentation and consumer acceptance. Microbiological analysis confirmed the presence and viability of total viable counts (TVC), highlighting its potential to promote gut health. Sensory evaluation results showcased 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 constitutes a significant advancement in functional foods and nutrition, offering a sustainable and health-promoting option for enhancing gut health and overall well-being. Future research endeavors may concentrate on optimizing formulation and processing parameters to enrich the nutritional profile, sensory attributes, and shelf stability of the product, along with conducting clinical studies to validate its efficacy in supporting gut health and wellness.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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