

From Ancient Grains to Modern Science: “Probiotic Potential in Traditionally Processed Indian Millets”

Dr. Anjana Shukla¹, Akella Sarvani², Pinky kumari³

¹Assistant Professor, Department of Botany

² Under Graduate Student

³ Under Graduate Student

St. Pious X Degree & PG College for Women (Autonomous), Hyderabad

Email Id: dr.anjanashukla@gmail.com

ABSTRACT

Millets, often described as ancient grains, have been an indispensable component of traditional Indian diets due to their high nutritional value and resistance to diverse environmental conditions (Amadou et al., 2013; Sarita & Singh, 2016). In the recent years, scientific interest has focused on the role of conventionally processed millets as functional and probiotic foods (Sharma & Niranjana, 2018). Indigenous processing techniques such as soaking, germination, malting, and natural fermentation significantly influence the nutritional composition and microbial profile of millet-based products (Shaikh et al., 2017). Fermented millet foods are known to support the growth of beneficial microorganisms, particularly lactic acid bacteria and yeasts, which exhibit probiotic-associated properties such as tolerance to acidic and bile conditions and incompatible activity against pathogenic microorganisms (Songré-Ouattara et al., 2013; Byakika et al., 2020). Traditional fermentation processes reduce anti-nutritional factors, including phytates and tannins, thus enhancing mineral bioavailability and protein digestibility. Millets also function as effective prebiotic substrates owing to their high content of dietary fibre, resistant starch, and bioactive phenolic compounds, which promote the growth and metabolic activity of beneficial gut microbiota (Sori et al., 2023). Furthermore, fermentation enhances the production of bioactive metabolites. These combined probiotic and prebiotic qualities suggest that traditionally processed millets act as natural symbiotic foods. In the present study, millet samples were collected from local markets and home-prepared sources, incubated at 37 °C, and subjected to serial dilution for bacterial isolation. Preliminary identification was performed using Gram staining, followed by biochemical characterization. The findings highlight the potential of traditionally processed Indian millets as sustainable, non-dairy foods for improving gut health.

KEYWORDS:

Indian millets, Traditional processing, Fermentation, Probiotics; Prebiotics, Micro biota.

INTRODUCTION

Millets are a group of small-seeded cereal grains belonging to the family *Poaceae*. They are well adapted to arid and semi-arid regions of Africa and Asia and occur in various sizes and colours depending on the species (Amadou et al., 2013). Global millet production was estimated at 27,827 metric tons in 2014, with Asia contributing around 95% of the total production (Sarita & Singh, 2016). India is the leading producer (11,066 metric tons), followed by China. In recognition of their nutritional and health benefits, the United Nations General Assembly, on the proposal of the Government of India, declared 2023 as the International Year of Millets under the theme “*Healthy Millets, Healthy People*.”

In India, millet consumption varies widely across regions. Higher consumption is reported in Gujarat (pearl millet and maize), Karnataka (finger millet), and Maharashtra (sorghum), while consumption remains low in rice-staple states such as Kerala, Odisha, West Bengal, and Tamil Nadu (Sarita & Singh, 2016). Although cereals contribute 70–80% of total dietary energy, millets account for only about 2% of caloric intake among urban Indians (6.7 g/day). Pearl millet (*Pennisetum glaucum*) constitutes approximately 40% of global millet production, followed by foxtail millet (*Setaria italica*), kodo millet (*Paspalum scrobiculatum*), and finger millet (*Eleusine coracana*). Based on cultivation extent, millets are broadly classified into major and minor millets (Amadou et al., 2013).

Millets as probiotics and prebiotics

Millets function as both prebiotic and probiotics, depending upon how they are prepared (Byakika et al., 2020).

Probiotics help to restore and sustain a healthy gut microflora, whereas prebiotics specifically encourage the growth of beneficial intestinal bacteria. Whole millets function as natural prebiotics, and fermented millet products are effective probiotic foods that enhance digestion and gut health (Sori et al., 2023). Foods based on millet that contain probiotics have demonstrated potential in managing diarrhoea, inflammatory bowel disease, diabetes, and in lowering the risk of colorectal cancer, thus establishing them as valuable functional foods (Chaudhary & Mudgal, 2020).

Foxtail millet-*Setaria italica* (L.) P. Beauv

Foxtail millet, a member of the Paniceae tribe (subfamily Panicoideae, Poaceae), was domesticated from green millet in northern China approximately 8,000 years ago (Sun et al., 2019). It is widely cultivated in semi-arid and arid regions of Africa, Asia, and the Americas due to its high nutritional value (starch, protein, dietary fiber, fats, vitamins, and minerals), low-glycaemic and hypolipidemic properties, minimal input requirements, and tolerance to biotic and abiotic stresses such as drought, salinity, and fungal diseases (Sharma & Niranjana, 2018).

Sorghum millet *Sorghum bicolor* (L.) Moench.

It is a tall, annual cereal grass with a robust morphology belonging to family Poaceae. It has a fibrous root system that helps in drought resistance. The stem is thick, erect, and can grow 1–4 meters tall. The leaves are long, narrow, and lanceolate with parallel venation. The inflorescence is a compact or loose panicle, bearing small spikelet that contain bisexual and sterile flowers. The grains are small, round, and vary in colour from white to red, brown, or black, depending on the variety. Sorghum is highly adaptable to arid and semi-arid regions due to its efficient water-use mechanism (Byakika et al., 2020).

Kodo millet (*Paspalum scrobiculatum* L.)

It originated in India and is believed to have been domesticated around 3,000 years ago. It is well-suited to tropical and subtropical regions, thrives on poor soils, and is widely cultivated in arid and semi-arid areas of India and Africa, particularly in Gujarat, Karnataka, Chhattisgarh, eastern Madhya Pradesh, and parts of Tamil Nadu. Kodo millet is an annual grass reaching about 90 cm in height, with grains ranging from light red to dark grey enclosed in a tough husk. Traditionally, it is consumed as porridge, flatbread, steamed couscous, or fried (Deshpande et al., 2015).

Pearl millet – *Pennisetum glaucum* (L.) R. Br.

Pearl millet (Poaceae) serves as a food source for millions residing in the semi-arid tropics, offering calories and playing a crucial role in food security within these areas (FAO and ICRISAT, 1996). This grain is utilized to create a diverse range of food items, such as cooked whole grains, both thin and thick porridges, weaning foods, and malted products intended for alcoholic beverages (Badau, 2006).

Finger millet- (*Eleusine coracana* L.)

It belongs to the "Poaceae" family, and it is available in red, brown, yellow, and white seed coats. It is a significant millet widely grown in India and Africa and is a staple food for a considerable portion of the people in these countries. It is well-known for its nutritional quality, which includes high amounts of dietary fibre, protein, and starch, as well as high mineral levels (Sarita & Singh, 2016). As the millets are gluten free, it has considerable potential in foods and beverages which can meet the growing demand for gluten free foods (Sharma & Niranjana, 2018).

METHODOLOGY

Samples of millet were collected from local retail stores and homemade sources; five samples of millets were chosen for analysis. Serial dilution was utilized to isolate bacterial colonies and reduce contamination under different seasonal conditions (Badau, 2006). The samples were combined with sterile water to create successive dilutions, which were subsequently inoculated onto nutrient agar plates using the pour plate technique and incubated at 37 °C for 24 hours. After incubation, bacterial growth was noted and documented. The process of serial dilution involved stepwise, constant-factor dilutions to attain logarithmic reductions in microbial concentration.

Identification of probiotic bacteria:

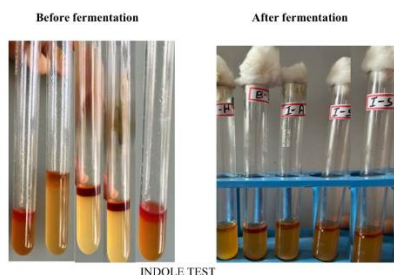
Serial dilution allowed systematic reduction of microbial concentration for bacterial colony isolation. Gram staining was carried out to identify bacterial type: heat-fixed smears were stained with crystal violet, treated with Gram iodine, decolorized with alcohol, counterstained with safranin, and examined microscopically to determine whether isolates were Gram-positive or Gram-negative. (Cappuccino & Sherman, 2014).

Biochemical characterization of bacteria

Indole Test

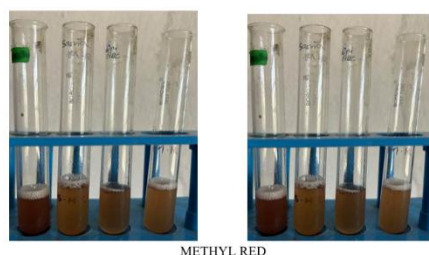
The indole assay was carried out to determine the production of indole by the test organisms isolated from millet samples. A peptone broth was created, comprising peptone (10 g), sodium chloride (5 g), and distilled water (100 mL), which was then sterilized at 121 °C for 15 minutes. After the sterilization

process, the broth was inoculated with the test organisms and incubated at 37 °C for 24 hours. Upon the conclusion of the incubation period.



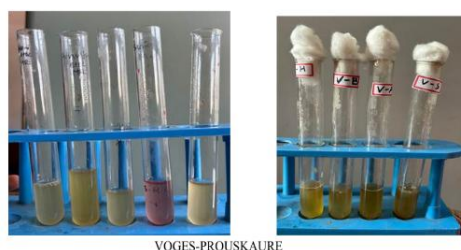
Methyl red test

The methyl red test was conducted to evaluate mixed-acid fermentation by bacterial isolates obtained from millet samples. Methyl red -VP broth was prepared and inoculated with the test organisms, followed by incubation at 37 °C for 24 hrs. After incubation, 5 ml of culture was transferred to sterile test tubes, and a methyl red indicator was added. The development of a stable red or yellow colour was observed to determine methyl red test positivity or negativity.



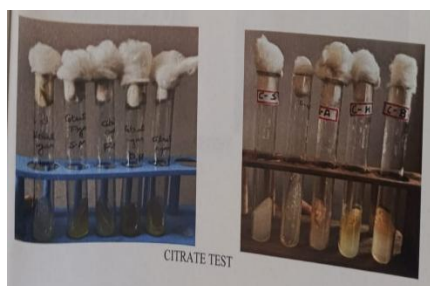
Voges-Proskauer test

The Voges–Proskauer (VP) test was performed to detect acetoin production by bacterial isolates obtained from millet samples. Methyl Red–Voges Proskauer (MR-VP) broth was inoculated with the bacterial cultures and incubated at 37 °C for 24 hours. Following incubation, Barritt's reagents A and B were added to the cultures, mixed gently, and observed for the development of a pink to red colour, indicating a positive VP reaction.



Citrate utilization test

The citrate utilization test was conducted to assess the capacity of bacterial isolates derived from millet samples to utilize citrate as their exclusive carbon source. Simmons citrate agar slants were inoculated with the test organisms and incubated at 37 °C for a duration of 24 hours. Citrate utilization was assessed by observing bacterial growth and a colour transition of the medium from green to blue.



CITRATE TEST

Catalase test

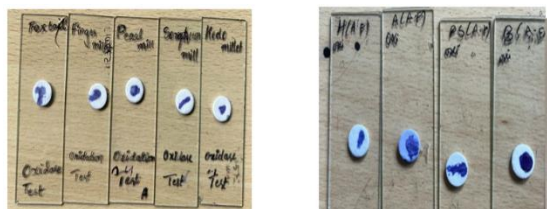
The catalase test was conducted to identify catalase activity in bacterial isolates derived from millet samples. A drop of 3% hydrogen peroxide was applied to the bacterial culture on a glass slide, and the rapid emergence of oxygen bubbles signified a positive reaction.



CATALASE TEST

Oxidase test

The oxidase test was performed to identify cytochrome c oxidase activity in bacterial isolates obtained from millet samples. Discs impregnated with oxidase reagent were inoculated with the test cultures, and the appearance of a colour change on the disc was monitored to assess oxidase positivity.



OXIDASE TEST

Starch hydrolysis test

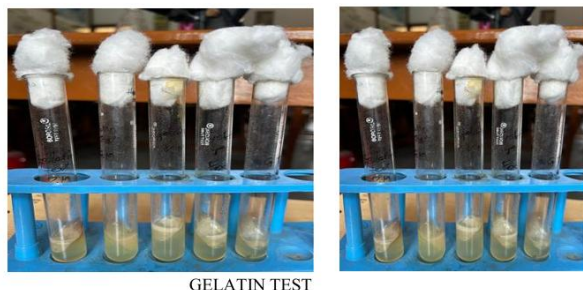
The starch hydrolysis test was conducted to assess the capability of bacterial isolates derived from millet samples to hydrolyse starch. Starch agar plates were inoculated with the test organisms and incubated at 37 °C for a duration of 24 hours. Subsequent the incubation period, iodine solution was introduced, and the appearance of a clear zone surrounding the colonies signified a positive reaction for starch hydrolysis.



STARCH TEST

Gelatine hydrolysis test

The gelatine hydrolysis test was conducted to estimate the production of gelatinase by bacterial isolates obtained from millet samples. Gelatine agar slants were inoculated and incubated for a duration of up to one week, and the liquefaction of the medium upon cooling signified a positive outcome.



GELATIN TEST

OBSERVATION

Biochemical Analysis of Millet Samples Before and After Fermentation:

Biochemical tests performed on five millet samples both prior to and following fermentation provide valuable insights into microbial activity and associated metabolic transformations (Songré-Ouattara et al., 2013). These analyses play a crucial role in detecting specific microorganisms and understanding their enzymatic functions, which collectively drive the fermentation process (Songré-Ouattara et al., 2013).

Before Fermentation:

The test results show that the raw millet samples contain certain microbes, but their biochemical activities are limited. The presence of biochemical reactions like Methyl Red, Citrate Utilization, and Starch Hydrolysis suggests that some bacteria are already present in the raw millet. Though, the microbial diversity is relatively low before fermentation.

Table 1: Table 2: Identification of bacterial colonies from the millet samples by biochemical tests before fermentation.

Table 1: Identifying the bacteria sample in millets before fermentation:

Biochemical Test	Pearl millet	Finger millet	Foxtail millet	Kodo millet	Sorghum millet
Indole	+	+	-	+	-
Methyl Red	-	-	+	-	+

Voges Proskauer	+	+	+	-	-
Citrate utilization	-	-	-	+	+
Catalase	+	+	+	-	+
Oxidase	-	+	-	+	-
Starch	-	+	+	-	+
Gelatine	+	-	-	+	+
Gram staining	-	+	+	-	+
Species	Aeromonas	Micro	Lacto	Pasteurella	Bacillus
	hydrophila	coccus	bacillus	sps	cereus

Table 2: Identifying the bacteria sample in millets before fermentation:

Isolates	Shape	Size	Colour	Species	Gram Reaction
1.Pearl millet	Rod	Small	Grayish	Aeromonas hydrophilia	-
2. Finger millet	Spherical	Small	Yellow	Micro coccus	+
3.Foxtail millet	Rod	Small	Yellow	Lacto bacillus	+
4.Kodo millet	Oval	Small	Grey	Pasteurilla	-
5.Sorghum	Rod	Large	Orange	Bacillus cereus	+

Fermentation leads to considerable biochemical and microbial transformations in millet samples, improving digestibility, nutrient bioavailability, and probiotic levels. Comparative biochemical tests conducted before and after fermentation reveal microbial succession and heightened enzymatic activity, which facilitates the degradation of complex macromolecules. The identification of microorganisms

including *Bacillus subtilis*, *Clostridium* spp., *Lactobacillus* spp., and *Providencia* spp. substantiates the active role of microbes in enhancing nutrient accessibility.

Table 3: Table 4: Identification of bacterial colonies from the millet samples by biochemical tests after fermentation.

Table 3: Identifying the bacteria sample in millets after the fermentation

Biochemical test	Pearl Millet	Finger Millet	Foxtail Millet	Kodo Millet	Sorghum Millet
Indole	+	-	-	+	+
Methyl red	-	+	-	+	-
Voges proskauer	+	-	+	-	+
Citrate utilization	+	+	+	-	-
Catalase	+	+	+	+	+
Oxidase	-	+	+	-	-
Starch	+	-	-	+	+
Gelatine	-	+	+	-	+
Gram staining	+	+	+	-	+
Species	Bacillus enterococcus	Bacillus megaterium	Bacillus subtilis	Clostridium sps	E coli or Providencia

Table 4: Identification of bacterial colonies from sample millets by biochemical test after fermentation

Isolates	Shape	Size	Colour	Species	Gram reaction
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1. Pearl millet	Rod	small	grayish	Bacillus enterococcus	-
2. Finger millet	Spherical	small	yellow	Bacillus megaterium	+
3. Foxtail millet	Rod	Small	Yellow	Bacillus subtilis	+
4. Kodo millet	Oval	small	grey	Clostridium	-
5.Sorghum millet	Rod	Large	Orange	E. coli	+

Bacterial Characterization of Millet Samples

The identification of bacterial colonies based on size, colour, and Gram reaction. The bacteria isolated before fermentation include *Aeromonas hydrophila*, *Micrococcus*, *Lactobacillus*, *Pasteurella*, and *Bacillus cereus*. After fermentation, the bacterial species shift to include *Bacillus enterococcus*, *Bacillus megaterium*, *Bacillus subtilis*, *Clostridium*, and *E. coli*. This shift indicates a transition in microbial diversity due to fermentation. Before fermentation, a mix of Gram-positive and Gram-negative bacteria is present. Though, after fermentation, more *Bacillus* species are found, along with *Clostridium* and *E. coli*, suggesting changes in the microbial environment due to fermentation.

Biochemical and Gram Staining Results

Biochemical analysis before and after fermentation revealed notable metabolic activity and shifts in bacterial populations. Catalase activity was detected in most samples, indicating the presence of aerobic or facultative anaerobic bacteria. Positive methyl red and Voges–Proskauer reactions after fermentation suggest mixed acid fermentation and acetoin production, reflecting metabolic diversification (Cappuccino & Sherman, 2014).

Citrate utilization and starch hydrolysis were positive, demonstrating the ability of bacteria to utilize citrate as a carbon source and degrade complex carbohydrates during fermentation. Indole, gelatine hydrolysis, and oxidase tests aided in bacterial differentiation and confirmed the presence of enzymatically active microorganisms.

Gram staining showed a predominance of Gram-positive bacteria in post-fermentation samples, particularly *Bacillus* species such as *Bacillus subtilis* and *Bacillus megaterium*, indicating their adaptive advantage under fermentation conditions.

CONCLUSION

This study isolated and screened probiotic bacteria from selected millets—sorghum, pearl millet, finger millet, foxtail millet, and kodo millet—revealing their potential as natural sources of beneficial microbes (Byakika et al., 2020). Serial dilution techniques enabled the successful isolation and culture of distinct strains on nutrient media under controlled conditions. Gram staining differentiated gram-positive from gram-negative bacteria, yielding key insights into their structural properties.

Biochemical tests further characterized the isolates' enzymatic and metabolic capabilities, critical for probiotic functionality. Assays for indole production, methyl red, Voges-Proskauer, citrate utilization,

catalase, oxidase, nitrate reduction, starch hydrolysis, and gelatine hydrolysis confirmed their ability to thrive in gastrointestinal environments, positioning them as promising probiotic candidates.

These findings establish millets as underexplored reservoirs of probiotics, extending their value beyond nutrition to gut health promotion (Sori et al., 2023). By applying robust methodologies, this research provides a foundation for developing millet-derived strains in functional foods and innovative health applications.

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