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EVALUATION OF PROBIOTIC VIABILITY AND GUT HEALTH BENEFITS IN DIFFERENT BEVERAGES MATRICS

Probiotic Viability

Gut Health

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"EVALUATION OF PROBIOTIC VIABILITY AND GUT HEALTH BENEFITS IN DIFFERENT BEVERAGES MATRICS"

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Abstract

This study was conducted to investigate the presence, identification, and functional potential of probiotic microorganisms in different fermented beverage matrices, with special emphasis on their role in supporting gut health. Fermented products such as wine and kanji were prepared using selected natural raw materials and allowed to undergo controlled fermentation under suitable environmental conditions. The prepared samples were subjected to microbiological analysis, including serial dilution, isolation, and characterization of bacterial strains. Identification was carried out using Gram staining and a series of biochemical tests such as catalase, citrate utilization, indole production, and enzyme activity assays.

In addition to identification, the study also evaluated the viability and adaptability of the isolated microorganisms under varying conditions, simulating their ability to survive in the gastrointestinal environment. The results revealed the presence of beneficial microbial strains showing positive biochemical activities, indicating their metabolic efficiency and potential probiotic nature. The fermentation process also contributed to improved nutritional quality and stability of the beverages.

Overall, the findings suggest that fermented beverages can serve as effective carriers of probiotic microorganisms and may contribute to maintaining gut microbial balance and enhancing digestive health. This study provides a scientific basis for the development of functional beverages with probiotic benefits.

Keywords:

Probiotics; Fermented beverages; Microbial viability; Gut health; Kanji; Wine fermentation; Microbial characterization; Functional beverages

1. Introduction

The concept of the human microbiota dates back to the early twentieth century, when scientists first recognized that the human body hosts a vast and diverse population of microorganisms, including bacteria, fungi, and viruses, particularly within the gastrointestinal tract and oral cavity [1]. These microbial communities are often described as a "hidden organ" due to their extensive metabolic capabilities and their profound influence on host physiology. The collective genetic material of these microorganisms significantly exceeds that of the human genome—by more than 150-fold—highlighting their biological importance [2].

Although the terms microbiota and microbiome are frequently used interchangeably, they have distinct meanings. Microbiota refers specifically to the community of living microorganisms present in a defined environment, whereas the microbiome encompasses not only these organisms but also their genomes, metabolites, structural components, and environmental interactions [3]. Thus, the microbiome represents a broader ecological and functional concept. This review primarily focuses on the functional role of microbiota, particularly the gut microbiota, in maintaining human health and contributing to disease processes.

Among all microbial communities, the gut microbiota is considered the most significant due to its central role in regulating multiple physiological functions. It contributes to digestion by fermenting indigestible dietary components, synthesizes essential vitamins such as vitamin K and B-complex vitamins, and plays a key role in immune system development and regulation [4][5]. The gut microbiota is predominantly composed of six major bacterial phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia. Among these, Firmicutes and Bacteroidetes are the most abundant and influential in metabolic processes [6]. In addition to bacteria, the gut ecosystem also includes fungi (mycobiota), viruses (including bacteriophages), and archaea, all of which contribute to the complexity and functionality of the microbial environment [7].

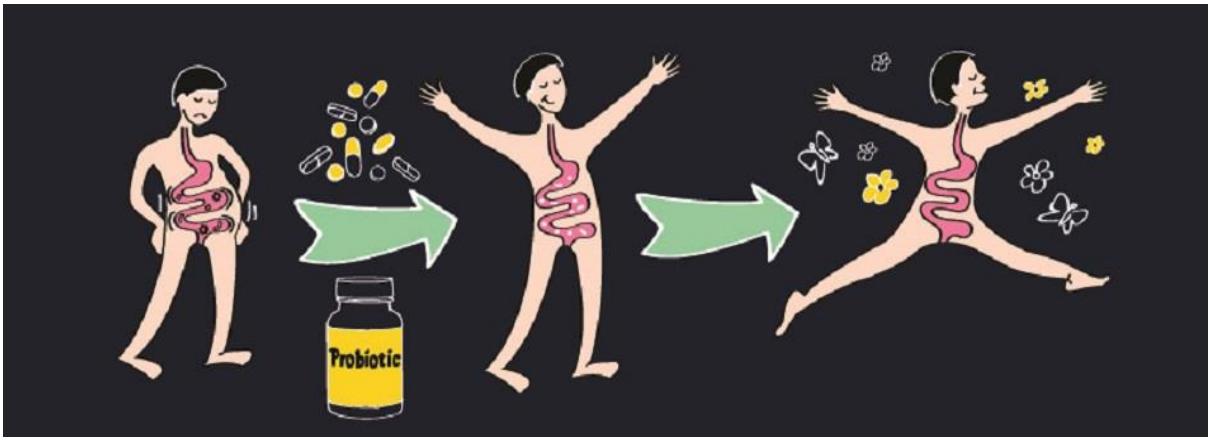
The human gastrointestinal tract harbors approximately 100 trillion microorganisms, making it one of the most densely populated microbial ecosystems known [8]. These microorganisms play a fundamental role in nutrient extraction, energy metabolism, and immune regulation [9]. Their metabolic potential is largely attributed to their diverse genetic repertoire, which encodes enzymes capable of breaking down complex carbohydrates, proteins, and lipids that are otherwise indigestible by human enzymes [10]. Additionally, the gut microbiota contributes to the biosynthesis of essential biomolecules, including amino acids, fatty acids, and vitamins, thereby supporting host nutrition and metabolic balance [11].

Beyond metabolic functions, gut microbiota also serves as a protective barrier against pathogenic microorganisms. It achieves this through competitive exclusion, production of antimicrobial compounds, and stimulation of host immune responses. Under healthy conditions, the gut microbiota exists in a stable and symbiotic relationship with the host, characterized by high diversity, resilience, and functional redundancy [12]. However, this balance is highly dynamic and can be influenced by various factors such as diet, age, environmental exposure, and the use of antibiotics or other medications. Disruptions in microbial composition, often referred to as dysbiosis, have been associated with a wide range of diseases, including inflammatory bowel disease, metabolic disorders, and immune-related conditions.

Probiotics are defined by the World Health Organization as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [13]. These beneficial microbes contribute to gut health

by enhancing microbial balance, improving digestion, and strengthening immune responses. Their mechanisms of action involve complex interactions with the host immune system, particularly through microbe-associated molecular patterns (MAMPs) that interact with pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) [14][15]. Activation of these receptors helps regulate immune responses, maintain intestinal barrier integrity, and prevent excessive inflammation [16]. However, dysregulation of these signaling pathways can lead to inflammatory diseases and compromised gut health [17][18].

The health benefits of probiotics have been widely demonstrated in both clinical and experimental studies. They have shown effectiveness in managing conditions such as antibiotic-associated diarrhea, acute gastroenteritis, inflammatory bowel disease, allergic disorders, and necrotizing enterocolitis. Probiotics are commonly delivered through fermented foods and beverages, where they must remain viable during processing and survive the harsh conditions of the gastrointestinal tract to exert their beneficial effects. Typically, a minimum viable count of 10^6 colony-forming units (CFU) per gram is required for therapeutic efficacy. While dairy-based products have traditionally been the primary carriers of probiotics, increasing consumer demand for plant-based alternatives has led to the development of non-dairy probiotic foods and beverages [19]. Importantly, probiotic effects are strain-specific, meaning that each strain must be evaluated individually for its functional properties and health benefits.



Probiotics – good bacteria

1.1 Fermentation of Wine

Fermentation is a key process in the production of many probiotic-rich foods and beverages. Wine fermentation is a biochemical process in which sugars present in fruit juices are converted into ethanol and carbon dioxide by yeast, primarily *Saccharomyces cerevisiae* [20]. In addition to ethanol production, fermentation generates secondary metabolites such as esters, higher alcohols, and organic acids, which contribute to the flavor, aroma, and overall quality of wine [21].

Wine fermentation typically occurs in two main stages. The first stage, known as primary fermentation, involves the rapid conversion of sugars into ethanol. The second stage, malolactic fermentation, involves the conversion of malic acid into lactic acid by lactic acid bacteria, which improves the taste, stability, and microbial safety of the final product. Furthermore, wine contains bioactive compounds such as polyphenols and antioxidants, which have been associated with potential health benefits, including anti-inflammatory and cardioprotective effects.



Ingredients used for fruit wine preparation

1.2 Fermentation of Kanji

Kanji is a traditional Indian fermented beverage prepared from black carrots, mustard seeds, salt, and water. Unlike wine, kanji undergoes natural lactic acid fermentation primarily driven by lactic acid bacteria such as *Lactobacillus plantarum* [22][23]. During fermentation, sugars are converted into lactic acid, leading to a decrease in pH, increased microbial stability, and the development of a characteristic sour taste. This process typically occurs over 3–5 days under ambient conditions and does not require the addition of starter cultures, as the necessary microorganisms are naturally present in the raw ingredients.

Kanji is considered a rich source of probiotics and is known for its beneficial effects on digestive health [24]. In addition to lactic acid bacteria, other microorganisms such as *Bacillus* species may also be present. While *Bacillus* species are not dominant in wine fermentation and may act as spoilage organisms at high concentrations, they can play a beneficial role in traditional fermented foods. Certain species, such as *Bacillus subtilis* and *Bacillus coagulans*, produce enzymes like amylases, proteases, and lipases that enhance nutrient availability and food quality. They also produce antimicrobial compounds such as bacteriocins and lipopeptides, which inhibit the growth of pathogenic microorganisms and contribute to food preservation [25][26]. Additionally, their ability to form spores allows them to survive harsh environmental conditions, making them promising candidates for probiotic and functional food applications.

2. Methodology

2.1 Study Design

The present study was conducted to evaluate the viability of probiotic microorganisms and their potential gut health benefits in different fermented beverage matrices, namely mixed fruit wine, black grape wine, and kanji. The investigation included preparation of fermented beverages, microbial isolation and identification, assessment of microbial viability, estimation of carbohydrate and ethanol content, biochemical characterization of isolates,

and phytochemical analysis of selected fruit components. Fermented beverages are recognized as effective carriers of beneficial microorganisms and bioactive compounds that contribute to gut health [27].

2.2 Materials

2.2.1 Raw Materials

Fresh fruits (apple, pineapple, orange peel, and grapes) were used for mixed fruit wine, while black grapes were used for grape wine. Kanji was prepared using beetroot, carrot, mustard seeds, black salt, and chili powder. Sugar and distilled water were also used. These materials were selected due to their high nutritional value and natural microbial load, which favor fermentation.

2.2.2 Culture Media and Reagents

Nutrient agar and MRS agar were used for microbial growth and lactic acid bacteria, respectively. Gram staining reagents (crystal violet, iodine, ethanol, and safranin), biochemical test reagents, and phytochemical reagents were also employed.

2.2.3 Equipment

The study utilized standard microbiological equipment, including an autoclave, incubator (37°C), laminar airflow cabinet, light microscope, pH meter, and sterile laboratory glassware.

2.3 Preparation of Fermented Beverages

2.3.1 Mixed Fruit Wine

Selected fruits were washed, cut, and crushed to extract juice, which was filtered through muslin cloth. Sugar was added to achieve 20–22 °Brix, and fermentation was carried out using *Saccharomyces cerevisiae* at 25–28°C for 5–7 days under anaerobic conditions. The fermented product was filtered and stored at 4°C [21].

2.3.2 Black Grape Wine

Black grapes were processed to obtain juice, which was adjusted for sugar content and pH (3.2–3.5). The must was inoculated with *Saccharomyces cerevisiae* and fermented at 25°C for 7–10 days. After fermentation, the wine was clarified and stored under refrigeration.

2.3.3 Kanji Preparation

Kanji was prepared by fermenting beetroot and carrot with mustard seeds, salt, and spices in water. The mixture was exposed to sunlight for 3–5 days for natural lactic acid fermentation. The final product was filtered and stored at 4°C.

2.4 Phytochemical Analysis (Apple)

Qualitative phytochemical tests were performed to detect bioactive compounds such as alkaloids, tannins, flavonoids, phenols, terpenoids, saponins, and steroids using standard methods including Mayer's, Wagner's, ferric chloride, Salkowski, and froth tests. These compounds contribute to antioxidant activity and nutritional value [28][29].

2.5 Microbial Isolation and Enumeration

2.5.1 Serial Dilution

Samples were serially diluted up to 10^{-6} to obtain countable microbial populations [30].

2.5.2 Spread Plate Method

Aliquots were spread on nutrient agar plates and incubated at 37°C for 24–48 hours. Colony counts were expressed as CFU/mL [31].

2.5.3 Streak Plate Method

Distinct colonies were sub-cultured to obtain pure isolates using the streak plate technique.

2.6 Gram Staining

Gram staining was performed to differentiate bacterial isolates. The presence of Gram-positive rod-shaped bacteria indicated *Bacillus* species, known for their probiotic potential and spore-forming ability [32].

2.7 Estimation of Carbohydrates (DNS Method)

Reducing sugars were estimated using the DNS method, where sugars react with 3,5-dinitrosalicylic acid to form a coloured compound measurable at 540 nm. Sugar concentration was determined using a standard glucose curve [33].

2.8 Estimation of Ethanol

Wine and Kanji

Ethanol content was estimated using the dichromate oxidation method. Ethanol is oxidized to acetic acid, producing a green-coloured complex measured at 600 nm. Concentration was calculated using a standard curve.

2.9 Biochemical Characterization

Isolates were identified using biochemical tests such as catalase, indole, citrate utilization, starch hydrolysis, sugar fermentation, nitrate reduction, MR-VP, casein hydrolysis, urease, and H_2S production. These tests determine enzymatic and metabolic activities for microbial identification [30].

2.10 Role of *Bacillus* Species in Gut Health

Bacillus species are widely recognized probiotics due to their ability to form endospores, enabling survival in harsh gastrointestinal conditions [32]. Studies have shown that *Bacillus coagulans* improves symptoms of irritable bowel syndrome [34], while *Bacillus subtilis* enhances gut microbiota balance and intestinal health [35].

Additionally, *Bacillus* species produce antimicrobial compounds that inhibit pathogens such as *E. coli* and *Salmonella* [36]. They also secrete enzymes that aid digestion and improve nutrient absorption [37]. Furthermore, these bacteria modulate immune responses and promote gut health by enhancing immunoglobulin production and reducing inflammation [38].

Overall, *Bacillus* species contribute to gut health through multiple mechanisms, including antimicrobial activity, enzyme production, immune modulation, and improved microbial balance [39].

3. Results

3.1 Phytochemical test:

S.No.	Phytochemical test	Method (Reagent used)	Observation	Result
1	Alkaloids	Mayer's test	No cream precipitate	-
2	Tannis	Ferric Chloride Test Gelatin test	dark blue/green color white precipitate	+ +
3	Steroids	Liebermann–Burchard Test	green coloration	+
4	Flavonoids	Alkaline reagent test Lead acetate test	Yellow color observed Yellow precipitate formed	+ +
5	Terpenoids	Salkowski test	Reddish-brown interface	+
6	Saponins	Froth test	Stable persistent foam	+
7	Phenolic	Ferric Chloride Test Lead Acetate Test	Bluish-green coloration White precipitate formed	+ +

3.2 Preparation of Fermentated Beverages Matrics

The prepared raw materials were processed to develop three main fermented beverage systems: **mixed fruit wine** and **black grape wine** and **kanji**. The ingredients were washed, crushed, and mixed under sterile conditions, followed by natural fermentation for a period of 7 days at room temperature.

Fermentation setup of mix fruit wine, black grape wine and kanji after incubation

3.3 Microbiological Isolation Techniques

3.3.1 Serial Dilution Method

Serial dilution was performed to reduce microbial load and obtain isolated colonies from fermented samples (mix fruit wine, black grape wine, and kanji). One milliliter of each sample was aseptically transferred into 9 mL sterile distilled water to achieve a 10^{-1} dilution. Subsequent dilutions were prepared up to 10^{-6} using the same procedure. All steps were conducted under aseptic conditions. Appropriate dilutions were selected for further plating on nutrient agar.

3.3.2 Spread Plate Method

The spread plate technique was employed to evenly distribute microbial cells on nutrient agar. A 0.1 mL aliquot from selected dilutions (10^{-3}–10^{-6}) was spread uniformly using a sterile L-shaped spreader. Plates were incubated at 37°C for 24 hours to allow colony formation.

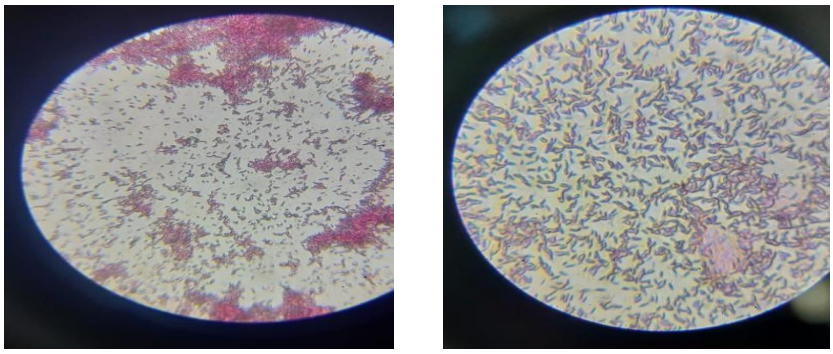
3.3.3 Streak Plate Method

Pure cultures were obtained using the streak plate method. Individual colonies from spread plates were streaked on nutrient agar using a sterile inoculating loop in quadrant patterns. Plates were incubated at 37°C for 24 hours to obtain isolated colonies.

3.4 Gram Staining

3.4.1 Microscopic Observation

Gram staining was performed on bacterial isolates and observed under oil immersion (100X). Clear visualization of cell morphology, arrangement, and staining characteristics was achieved.



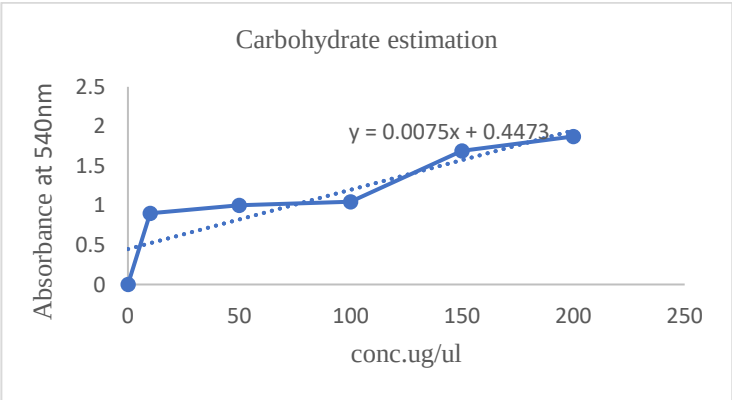
Microscopic view of Gram-stained bacterial cells

Overall, the isolates were identified as rod-shaped bacteria, including both Gram-positive and Gram-negative types, suggesting the presence of Bacillus and related species.

3.5 Estimation of Carbohydrate by DNS Method

The absorbance values of the test samples were recorded under the same conditions and compared with the standard curve to determine the carbohydrate concentration. The results obtained confirm the presence of reducing sugars in the samples.

The standard graph plotted between glucose concentration and absorbance was used for the quantitative estimation of carbohydrate content.



Conc.	Absorbance
0	0
10	0.900
50	1.002
100	1.047
150	1.691
200	1.872

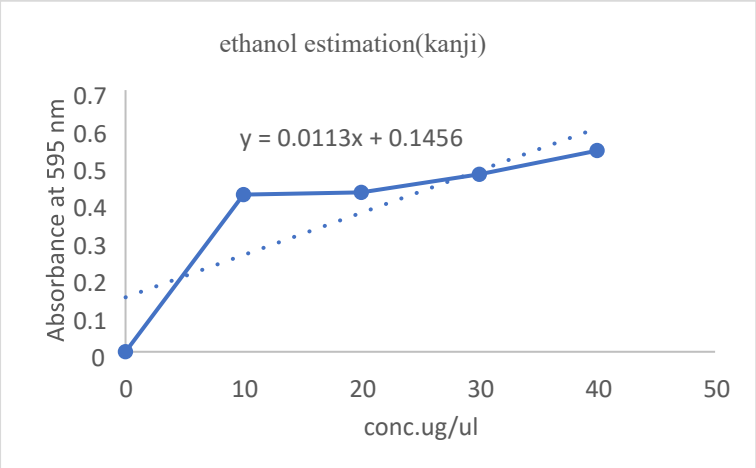
Standard curve of glucose for carbohydrate estimation

3.6 Estimation of Ethanol Content in Fermented Beverages

The ethanol content in fermented beverages (mix fruit wine, black grape wine, and kanji) was estimated using standard analytical methods. The samples were first filtered to remove suspended particles and then subjected to quantitative analysis.

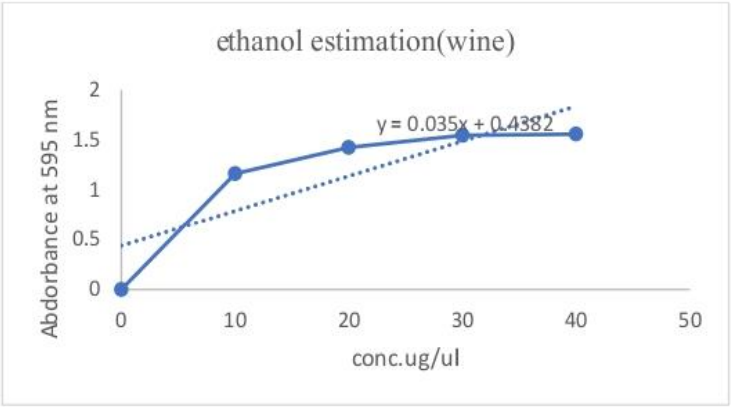
The results indicated variation in ethanol content among the different fermented samples. Fruit wine samples showed relatively higher ethanol levels compared to kanji, which exhibited lower alcohol content due to shorter fermentation duration.

The ethanol concentration obtained from the standard curve confirmed successful fermentation and production of alcohol in the samples.



Conc.	absorbance
0	0
10	0.42
20	0.426
30	0.475
40	0.538

Standard ethanol calibration curve for estimation of alcohol content



Conc.	Absorbance
0	0
10	1.162
20	1.424
30	1.545
40	1.557

Standard ethanol calibration curve for estimation of alcohol content

3.7 Biochemical Test:

Biochemical test	Mix fruit wine	Black grapes wine	Kanji
Casein test	+	+	+
Amylase test	+	+	+
H2S	-	-	-
Sugar fermentation test	+	+	+
Urease test	-	-	-
Catalase test	+	+	+
Nitrate test	-	-	-
Citrate test	+	+	+
Indole test	+	+	+
MR	-	-	-
MR-VP	-	-	-

3.8 Comparison of beverage samples

Parameter	Mixed fruit wine	Black Grapes wine	Kanji
Type	Alcoholic	Alcoholic	Non-Alcoholic
Raw Materials	Mixed fruits	Black grapes	Beetroot, carrot, spices
Fermentation	Yeast-dominant	Yeast-dominant	LAB-dominant
Alcohol Content	Moderate	High	Negligible
Microbial Growth	Moderate	Low	High
Probiotic Viability	Moderate	Low	High
Reason	Some ethanol inhibition	High ethanol inhibits microbes	Favorable acidic environment
Gut Health Benefit	Moderate	Low	High

4. Discussion

The present study evaluated the preparation, microbial characteristics, and biochemical properties of fermented beverages, namely mix fruit wine, black grape wine, and kanji. The results demonstrate that raw materials, fermentation conditions, and microbial activity significantly influence the quality and functional potential of these beverages.

The selected substrates supported effective fermentation within 7 days. Wine samples showed alcoholic fermentation with higher ethanol production, whereas kanji exhibited lactic acid fermentation with lower ethanol and higher acidity.

Microbiological techniques confirmed the presence of diverse and viable microbial populations. Gram staining revealed both Gram-positive and Gram-negative rod-shaped bacteria, with Gram-positive rods predominating in mix fruit wine and kanji.

Carbohydrate estimation confirmed the presence of residual reducing sugars, while ethanol analysis validated fermentation efficiency. Biochemical tests indicated metabolically active isolates with positive enzymatic activities and absence of harmful by-products.

Overall, all beverages showed beneficial microbial activity and good fermentation characteristics. Kanji demonstrated higher probiotic potential, while wine samples exhibited greater ethanol production, highlighting their nutritional and functional importance.

5. Conclusion

The present study successfully demonstrated the preparation and evaluation of fermented beverages as potential carriers of probiotic microorganisms. Fermented products such as wine and kanji were developed using natural raw materials and subjected to controlled fermentation, which supported the growth and activity of beneficial microbial populations. Microbiological analysis, including isolation, Gram staining, and biochemical characterization, confirmed the presence of bacteria exhibiting important enzymatic activities such as catalase and citrate utilization, indicating their metabolic capability and adaptability.

The results further revealed that these microorganisms were able to survive under varying environmental conditions, suggesting their potential to withstand stress factors similar to those encountered in the gastrointestinal tract. The fermentation process not only enhanced microbial growth but also improved the nutritional and functional properties of the beverages. Differences observed between beverage matrices indicated that the composition of the substrate plays a significant role in supporting probiotic viability and activity.

Overall, this study highlights the importance of fermented beverages as natural, cost-effective, and functional sources of probiotics. The findings suggest that regular consumption of such products may contribute to maintaining gut microbial balance and improving digestive health. Furthermore, this work provides a foundation for future research focused on molecular identification of strains and development of optimized probiotic formulations for enhanced health benefits.

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EVALUATION OF

PROBIOTIC VIABILITY

AND

GUT HEALTH BENEFITS

IN DIFFERENT BEVERAGES MATRICS

KEY HIGHLIGHTS

- Probiotic viability in diverse beverage matrices
- Impact on gut microbiota and digestive health
- Evaluation of health benefits and functional outcomes
- Reliable methods for viability and quality analysis
- Insights for innovation and future probiotic beverages

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