IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402, p- ISSN: 2319-2399.Volume 20, Issue 6 Ser. 1 (June 2026), PP 15-31
www.iosrjournals.org

Microbial Identification and Metabolite Profiling of Tibicos (Water Kefir) Fermentation Broth: A Preliminary Study for Functional Beverage Development

Hsiao-Han Liu[1], Shun-Po Tu[2], Tsung-Chiao Hsu[2]
Faculty of Department of Medical Science and Biotechnology, College of Medical Technology, I-Shou University, Kaohsiung, Taiwan, ROC; 2. Master Program of Department of Medical Science and Biotechnology, College of Medical Technology, I-Shou University, Kaohsiung, Taiwan, ROC)

Abstract:
Background*: Tibicos, also known as sugar kefir grains, are symbiotic assemblies of lactic acid bacteria and yeasts embedded in a polysaccharide matrix. While traditionally consumed as a health drink to improve metabolism and lower blood pressure, scientific validation of its microbial composition and metabolite functions remains limited. This study aims to characterize the microbial diversity and metabolite changes during Tibicos fermentation and evaluate its preliminary physiological effects on human health for functional beverage development.*
Materials and Methods*: Tibicos, also known as sugar kefir grains, are symbiotic assemblies of lactic acid bacteria and yeasts embedded in a polysaccharide matrix. While traditionally consumed as a health drink to improve metabolism and lower blood pressure, scientific validation of its microbial composition and metabolite functions remains limited. This study aims to characterize the microbial diversity and metabolite changes during Tibicos fermentation and evaluate its preliminary physiological effects on human health for functional beverage development.*
Results*: Microbial analysis identified six potential bacterial strains belonging to the *Bacillus* genus (including *B. circulans and B. oceanisediminis) and three yeast strains (Sporobolomyces koalae, Meyerozyma guilliermondii, and Aureobasidium pullulans). HPLC results revealed that fermentation for 48 hours significantly increased concentrations of lactic acid, malate, citrate, phosphate, and proteins, while carbohydrate levels decreased. The grains' weight increased by 45% after 48 hours of culture. In the human trial, a significant reduction in visceral fat was observed ($P < 0.05$), alongside reported improvements in insomnia, constipation, and overall metabolic comfort.*
Conclusion: *The Tibicos fermentation broth is a rich source of beneficial metabolites and diverse probiotic microbes. The significant increase in organic acids like lactic and citric acid contributes to improved digestive and metabolic health. These findings provide a scientific basis for the potential of Tibicos as a standardized functional health beverage.*
Key Word*: Tibicos, 16S/18S rDNA sequencing, HPLC, Organic acids, Visceral fat, Functional beverage.*

Date of Submission: 08-06-2026 Date of Acceptance: 19-06-2026

I. Introduction

Tibicos, also known as Sugar Kefir Grains, Water Kefir, or "Brown Sugar Bacteria" in localized contexts, is a symbiotic assembly of various microbial strains embedded within a white, semi-transparent polysaccharide matrix[1]. These grains typically consist of a complex community of lactic acid bacteria (such as *Lactobacillus* and *Leuconostoc*) [2]and yeasts (such as *Saccharomyces* and *Candida*)[3]. Unlike traditional milk kefir, which is cultured in dairy, Tibicos is fermented in non-dairy sugar solutions, typically using brown sugar or sucrose as a carbohydrate source[4, 5]. This characteristic makes it an ideal candidate for the development of probiotic functional beverages suitable for vegan populations and those with lactose intolerance[6].

Historically, Tibicos has been consumed for centuries as a traditional health tonic. Folklore suggests that the grains were brought from the East to Mexico, where they were distributed freely to help populations in remote areas with limited medical resources. In folk medicine, Tibicos fermentation broth is rumored to alleviate a wide range of ailments, including insomnia, hypertension, rheumatism, and metabolic disorders[5]. However, despite its

widespread traditional use, most of these health claims have historically lacked rigorous scientific validation and standardized quality control measures.

The motivation for this study stems from the increasing demand for health-promoting foods in modern society, where fast-paced lifestyles and high stress levels have led to a rise in metabolic syndromes and chronic diseases. While the market for functional beverages is expanding rapidly[3, 7], products that are scientifically proven to enhance physiological functions remain limited. Preliminary observations in our laboratory have indicated that consistent consumption of Tibicos broth may lead to improvements in blood pressure and metabolic markers. Yet, the precise microbial composition and the specific metabolite changes during fermentation—such as the production of organic acids and proteins—require detailed academic evidence to support its commercialization and clinical application.

Therefore, this research aims to provide a comprehensive scientific foundation for Tibicos as a functional beverage. The objectives are three-fold:

1. Microbial Identification: Utilizing molecular biological techniques, including 16S rDNA and 18S rDNA sequencing, to precisely identify the bacterial and yeast strains within the Tibicos grains.
2. Metabolite Profiling: Employing High-Performance Liquid Chromatography (HPLC) to quantify the changes in organic acids (such as lactic acid, malic acid, and citric acid), proteins, and carbohydrates during the 48-hour fermentation process.
3. Physiological Evaluation: Conducting a pilot human study involving 30 subjects to monitor the effects of Tibicos consumption on weight, BMI, visceral fat, and subjective health indicators.

By establishing the relationship between the microbial community and its metabolic output, this study seeks to demonstrate the value of Tibicos as a standardized, scientifically-backed health beverage that can contribute to global public health.

II. Material And Methods

A. Preparation of Tibicos Fermentation Broth

The Tibicos grains used in this study were provided by a specialized cultivation facility in Tainan, Taiwan[8]. The fermentation medium was prepared by dissolving brown sugar in filtered water at a fixed ratio of 15g (1.5 tablespoons) of sugar per 200cc of water. For each batch, 35g (3 tablespoons) of Tibicos grains were added to the solution in a glass container. The containers were covered with breathable plastic wrap to allow air circulation and incubated at a controlled room temperature of 25°C. Fermentation samples were collected at 0, 24, and 48-hour intervals for analysis. After fermentation, the broth was separated from the grains using a plastic strainer and stored at 4°C in glass containers to prevent acidic erosion.

B. Microbial Identification

The microbial composition of the Tibicos grains was analyzed using molecular biological techniques targeting both bacteria and yeasts.

Bacterial Identification: Genomic DNA was extracted from the grains. The 16S rDNA region was amplified via Polymerase Chain Reaction (PCR) using the primer sets 533R and 341Fgc[9]. The amplified fragments were purified and sequenced, with the results aligned against the NCBI database (Bacteria and Archaea) for strain identification.

Yeast Identification: Fungal colonies were isolated and purified using the four-quadrant streak method on Sabouraud Dextrose Agar (SDA). The 18S rDNA region was amplified using PCR with primers FR1 and NS1[10]. Following gel purification and sequencing, the strains were identified through NCBI BLAST alignment (Nucleotide collection).

Morphological Observation: Gram staining was performed to observe the microbial populations and determine the Gram-reactivity of the symbiotic community under a light microscope[11].

C. Metabolite and Chemical Analysis

Organic Acids and Ions: Metabolite profiling of the broth was conducted using High-Performance Liquid Chromatography (HPLC). The system utilized a Dionex IonPac® AS11 (2 × 250 mm) column with a 5-100 mM NaOH gradient mobile phase. Samples were filtered through a 0.2 μm nylon membrane prior to injection. Conductivity detection was employed to quantify lactic acid, malate, citrate, phosphate, and other ions[12].

Protein Quantification: Protein concentrations in the 0, 24, and 48-hour fermentation broth were measured using the Bradford method. A standard curve was established using Bovine Serum Albumin (BSA), and absorbance was recorded at OD595[13].

Carbohydrate Quantification: Water-soluble carbohydrate levels were determined using the phenol-sulfuric acid chemical colorimetric method, with absorbance measured at OD492[14].

D. Human Pilot Study and Physiological Assessment

A pilot study was conducted involving 30 human subjects (aged 45 and above) recruited from Kaohsiung City via simple random sampling[5].

Experimental Design: A four-week single-blind crossover design was implemented. For the first two weeks, subjects consumed 200 mL of pure brown sugar water daily (control group). For the subsequent two weeks, subjects consumed 200 mL of Tibicos fermentation broth daily (experimental group).

Physiological Measurements: Body weight, BMI, body fat, and visceral fat were measured using an OMRON HBF-370 multi-function scale. Blood pressure and pulse were recorded using an OMRON HEM-7011 arm-type electronic monitor. Measurements were taken weekly after dinner to minimize diurnal variation.

Subjective Evaluation: Subjects completed a Likert-scale (1-5) health questionnaire weekly to assess subjective changes in sleep quality, metabolism, and overall physical comfort across six dimensions (e.g., gastrointestinal, neurological).

E.Statistical analysis

All quantitative data were analyzed using SPSS Statistics software (versions 17.0 and 20.0). Physiological changes were evaluated using the paired-sample T-test to determine significance ($P < 0.05$). For questionnaire data, Cronbach's alpha was used to assess reliability[15], while construct validity was confirmed through Factor Analysis, Kaiser-Meyer-Olkin (KMO > 0.7) measures, and Bartlett's test of sphericity[15, 16].

III. Result

3.1 Tibicos Grain Weight During Fermentation (0, 24, 48 hr)

To monitor the growth of Tibicos grains during fermentation in brown sugar water, the weight of Tibicos grains was recorded at 0, 24, and 48 hours. As shown in **Table 1** and **Figure 1**, the weight of Tibicos grains increased progressively over time: from 7.31 g at 0 hr, to 8.91 g at 24 hr, and to 10.67 g at 48 hr. As illustrated in **Figure 2**, the weight at 24 hr increased by approximately 1.2-fold, and the weight at 48 hr increased by approximately 1.45-fold relative to the initial weight[12].

Table 1. Weight measurements of Tibicos grains cultivated in brown sugar water at 0, 24, and 48 hours.

hr	tibicos weight(g)	add weight(g)
0	7.31	0
24	8.91	1.6
48	10.67	3.36

Figure 1. Changes in Tibicos grain weight at 0, 24, and 48 hours.

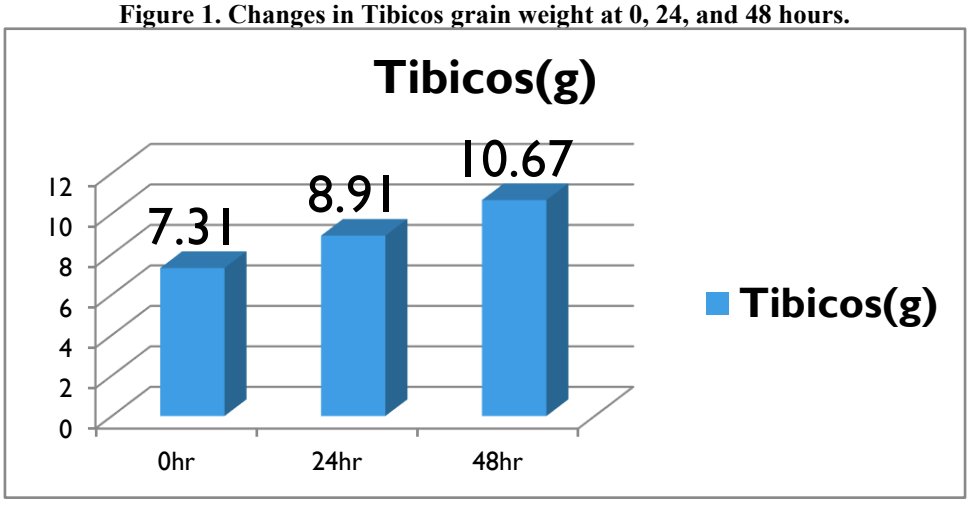
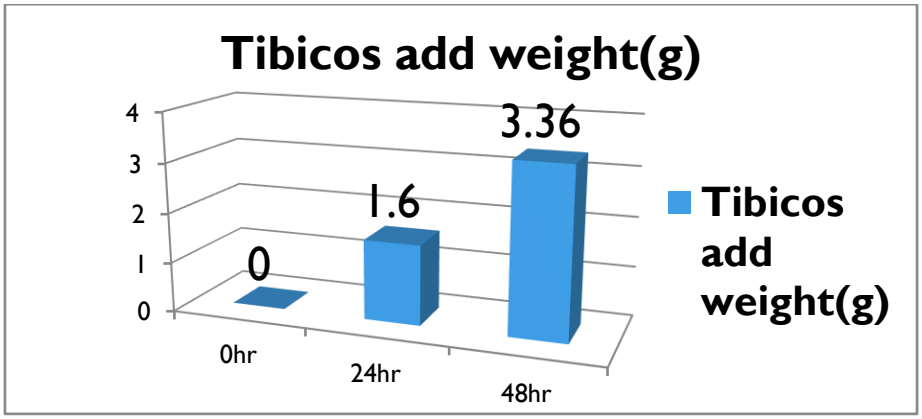


Figure 2. Fold change in Tibicos grain weight at 0, 24, and 48 hours.



3.2 Gram Staining of Tibicos

Gram staining was performed to determine the staining characteristics and morphological diversity of microorganisms within Tibicos[17]. Microscopic observation revealed a diverse microbial community, including rod-shaped bacteria (bacilli; **Figures 3,**), streptococci (**Figure 4**), cocci (**Figure 5**), and budding yeast cells (**Figure 6**). All microorganisms observed under the oil-immersion lens (1000×) and at 400× magnification stained purple-violet, confirming that the predominant organisms in Tibicos are **Gram-positive**(**Figure 7,8**). These results further demonstrate that Tibicos constitutes a **mixed microbial consortium**.

Figure 3. Gram-positive rod-shaped bacteria (bacilli) observed at 1000× oil-immersion magnification; cells stained purple-violet.

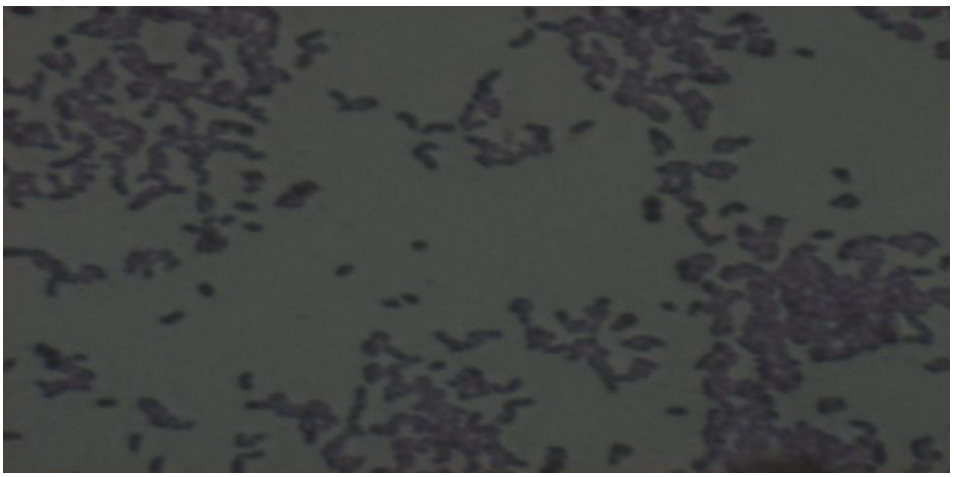


Figure 4. Gram-positive streptococci (chain-forming cocci) observed at 1000× oil-immersion magnification; cells stained purple-violet.

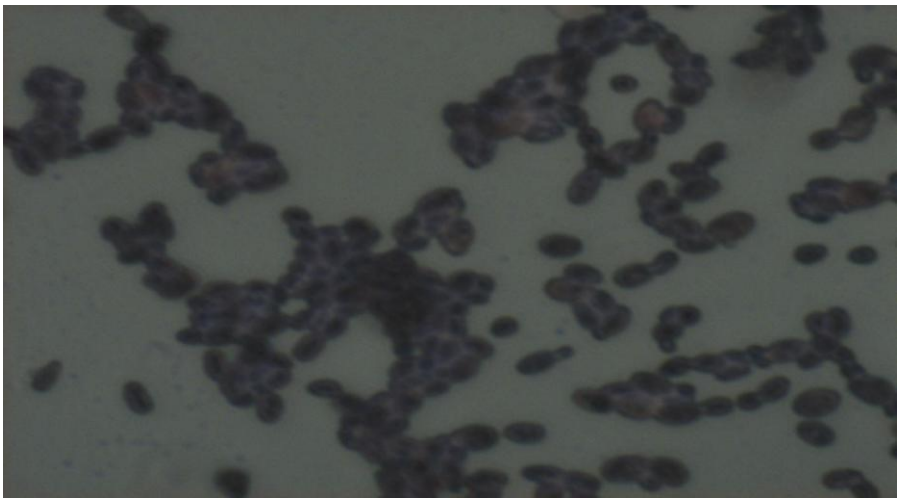


Figure 5. Gram-positive cocci observed at 400× magnification; cells stained purple-violet.

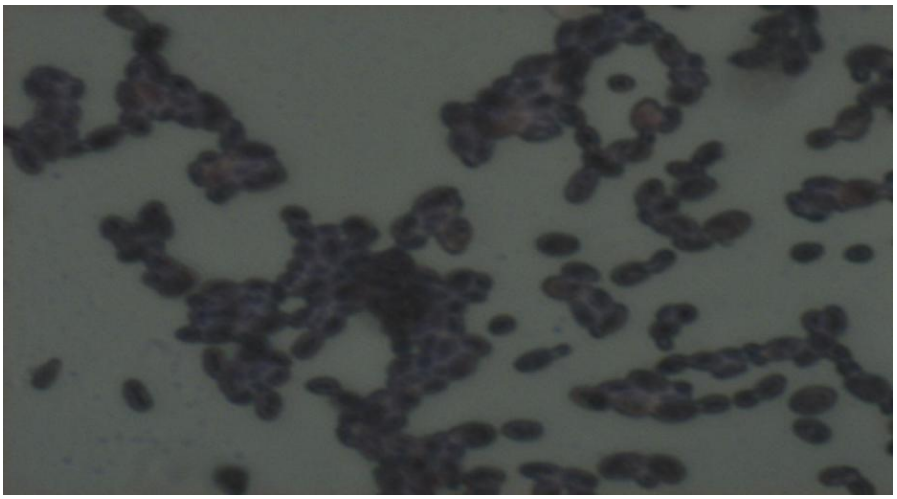


Figure 6. Gram-positive yeast cells observed at 1000× oil-immersion magnification; cells stained purple-violet.

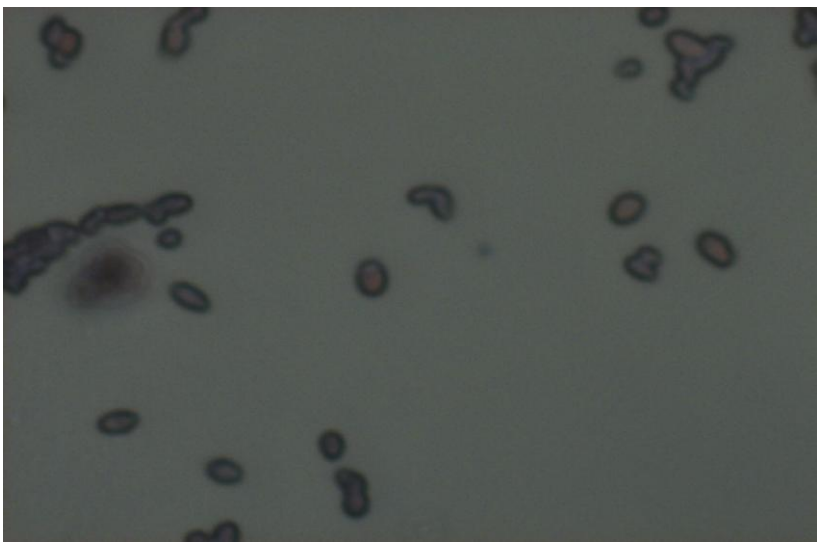


Figure 7. Gram-positive rod-shaped bacteria observed at 1000× oil-immersion magnification; cells stained purple-violet.

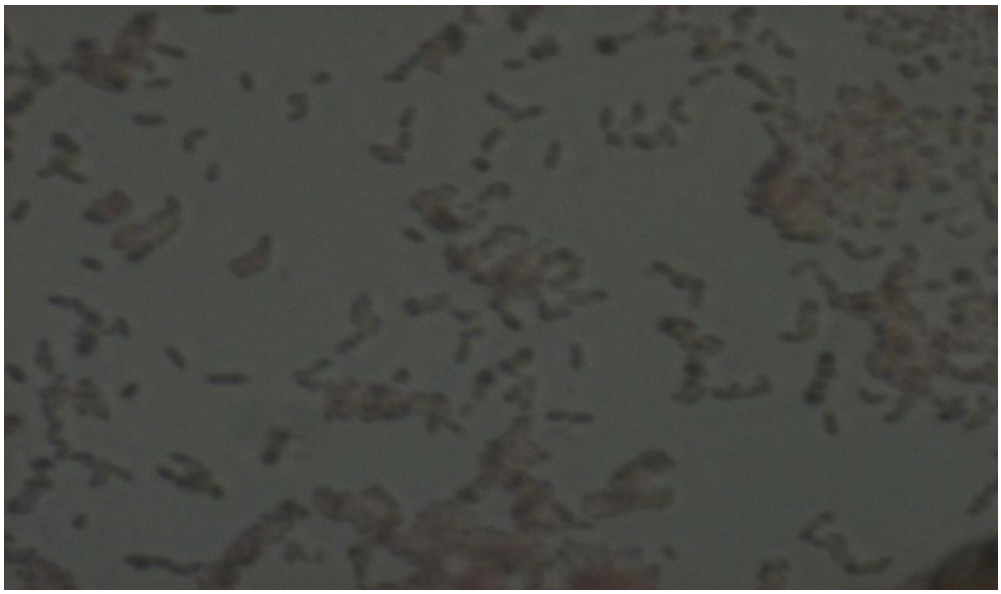
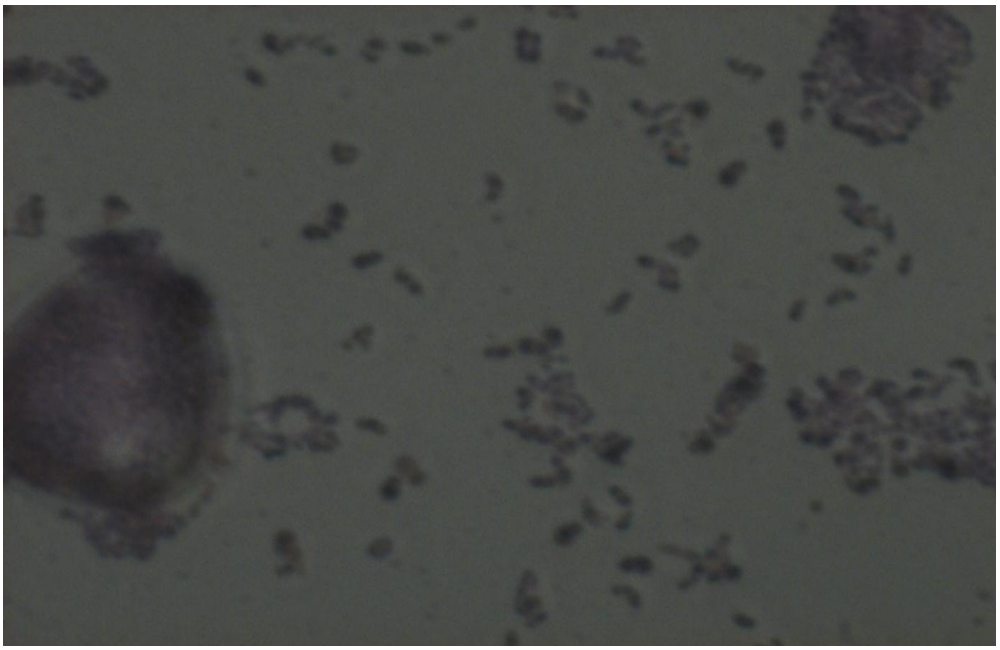


Figure 8. Gram-positive rod-shaped bacteria observed at 1000× oil-immersion magnification; cells stained purple-violet.

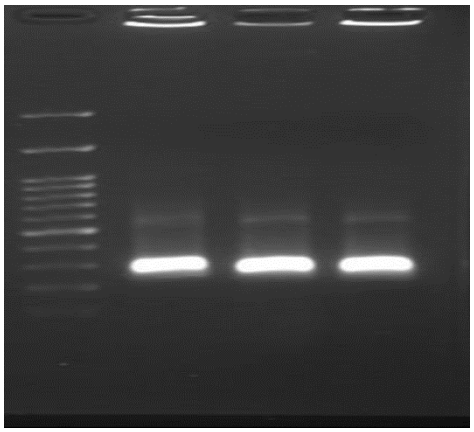


3.3 Bacterial Species Identification by 16S rDNA Molecular Analysis

Molecular Identification of the Microbial Community
Molecular analysis utilizing 16S and 18S rDNA sequencing identified a complex biodiversity within the grains.

1. **Bacterial Species:** Analysis of 16S rDNA (200-300 bp fragments) revealed six distinct strains from the ***Bacillus* genus**, notably *B. circulans*, *B. eiseniae*, and *B. oceanisediminis*6more_horiz.
2. **Yeast Species:** 18S rDNA sequencing identified three primary yeast strains: ***Sporobolomyces koalae***, ***Meyerozyma guilliermondii***, and ***Aureobasidium pullulans***.

Bacterial identification was performed using PCR amplification of the 16S rDNA gene with primer pairs 533R/341Fgc and 533R/341F, followed by DGGE separation and NCBI BLAST comparison[9]. As shown in



Figures 9 and 10, PCR-amplified fragments were detected in the 200–300 bp range on agarose gel electrophoresis, indicating that Tibicos harbors **at least six distinct bacterial strains**.

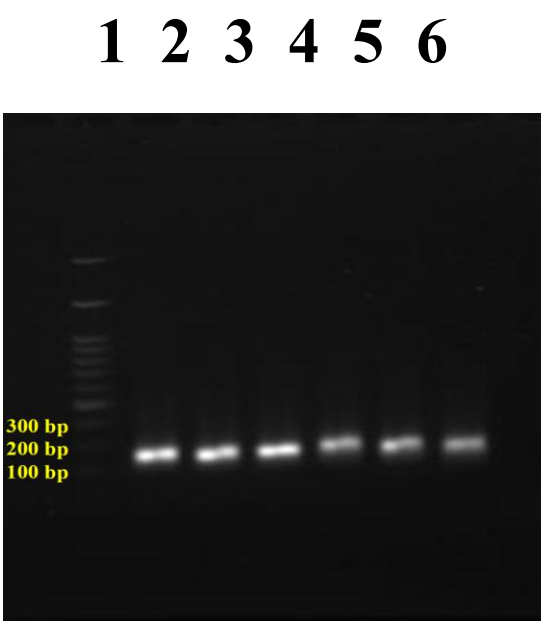


Figure 10. Agarose gel electrophoresis of PCR products amplified using primers 533R and 341Fgc; target bands are visible between 200 and 300 bp.

BLAST alignment results for each of the six bacterial strains are summarized in **Table 2** and described below:

Table 2. Summary of 16S rDNA BLAST identification results for the six bacterial strains isolated from Tibicos.

Strain	Most Probable Identification	Reference Sequence ID
Strain 1	*Bacillus circulans*	NR_118445.1
Strain 2	*Bacillus eiseniae*	NR_108906.1
Strain 3	*Bacillus oceanisediminis*	NR_118440.1
Strain 4	*Bacillus atrophaeus*	NR_075016.1
Strain 5	*Bacillus siralis*	NR_118440.1
Strain 6	*Bacillus massiliosenegalensis*	NR_125590.1

.

3.4 Yeast Species Identification by 18S rDNA Molecular Analysis

Tibicos grains were plated onto SDA medium using the four-quadrant streak method. As shown in **Figures 11 and 12**, colony observation from both the reverse and obverse sides of the SDA plates revealed three morphologically distinct yeast colony types: a **pink-colored** strain (designated *Tibicos 1*), an **orange-colored** strain (*Tibicos 2*), and a **white-colored** strain (*Tibicos 3*), confirming that Tibicos contains **at least three distinct yeast strains**.

Figure 11. Reverse side of SDA plate showing four-quadrant streak culture of Tibicos; pink and orange colonies are visible.

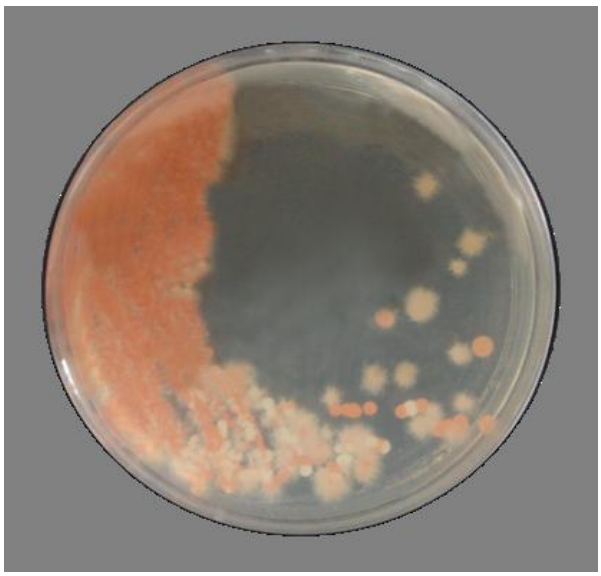
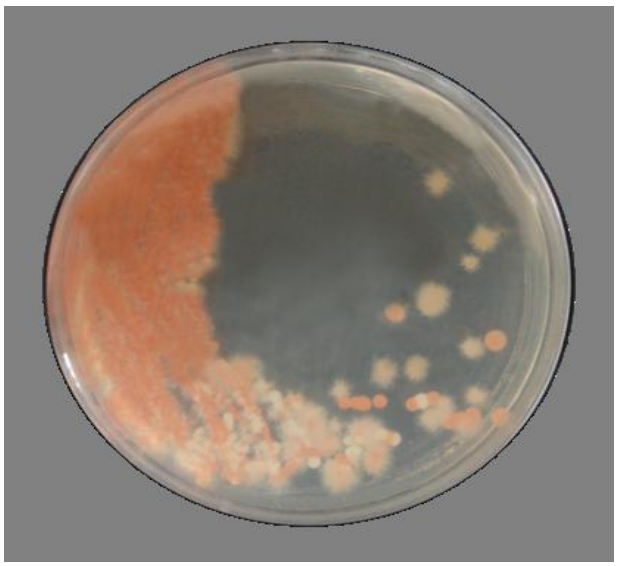
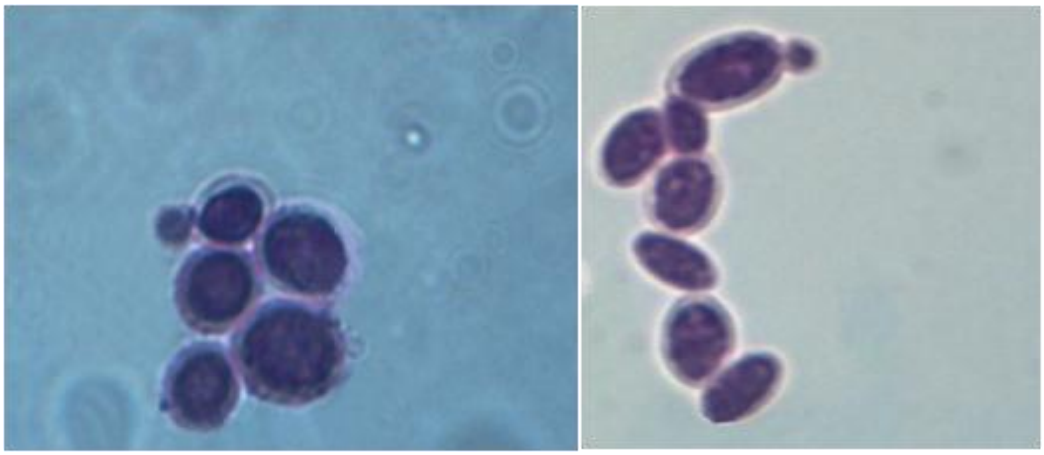


Figure 12. Obverse side of SDA plate showing four-quadrant streak culture of Tibicos; white colonies are clearly visible.



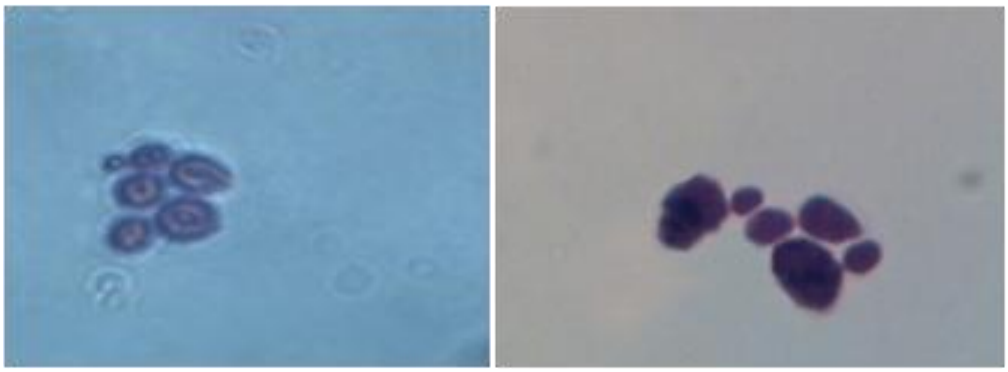
Gram staining of each purified colony revealed the following:

Tibicos 1 (**Figures 13a.b**): Both the pre-isolation smear and the purified colony exhibited morphology and size consistent with **Gram-positive budding yeast** (*Saccharomyces*-type budding yeast).

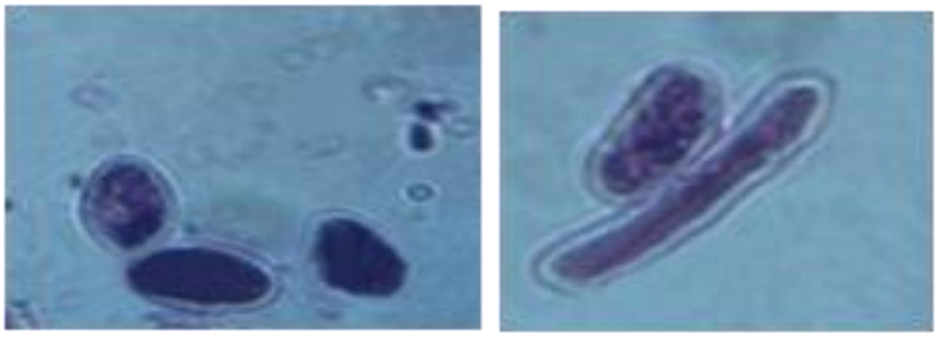


Figures 13a.b

Tibicos 2 (**Figures 14 a, b**): Both pre- and post-isolation smears were morphologically identical, confirming **Gram-positive budding yeast** morphology.



Tibicos 3 (**Figures 15 a,b**): Under 100× oil-immersion observation, budding yeast morphology (Figures 50–51) as well as hyphal (mycelial) morphology (Figures 52–53) were observed, both staining as **Gram-positive**.



PCR amplification of chromosomal DNA using the 18S rDNA primer pair (FR1/NS1) yielded target bands at approximately **1.5 kb and 3.0 kb**[10], as confirmed in **Figures 16 a, b**.

M T3 T2 T1 N T2 T3 N M

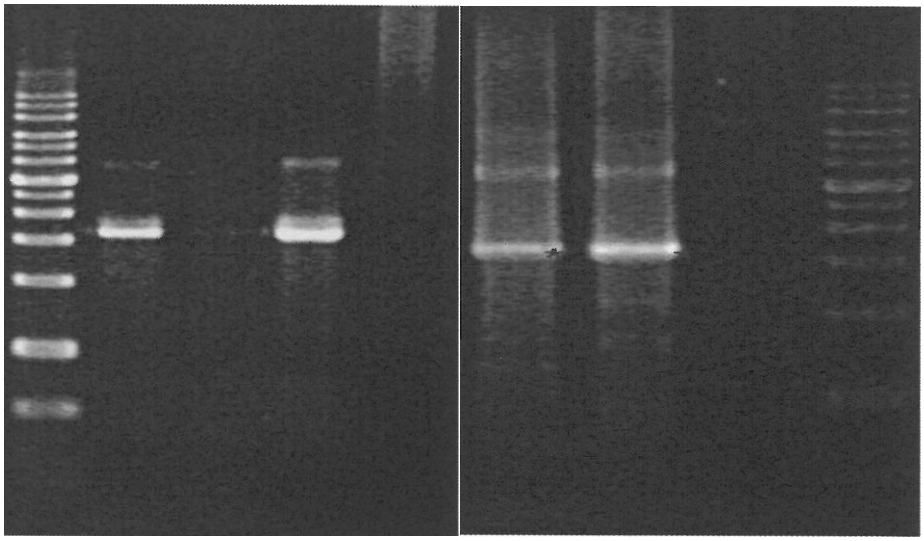


Table 3. Summary of 18S rDNA BLAST identification results for the three yeast strains isolated from Tibicos.

Strain	Colony Color	Morphology	Most Probable Identification
Tibicos 1	Pink	Budding yeast (Gram-positive)	*Saccharomyces cerevisiae*
Tibicos 2	Orange	Budding yeast (Gram-positive)	*Meyerozyma guilliermondii*
Tibicos 3	White	Budding yeast + Hyphae (Gram-positive)	*Aureobasidium pullulans*

3.5 Ion Liquid Chromatography (IC) Analysis of Organic Acids and Anions

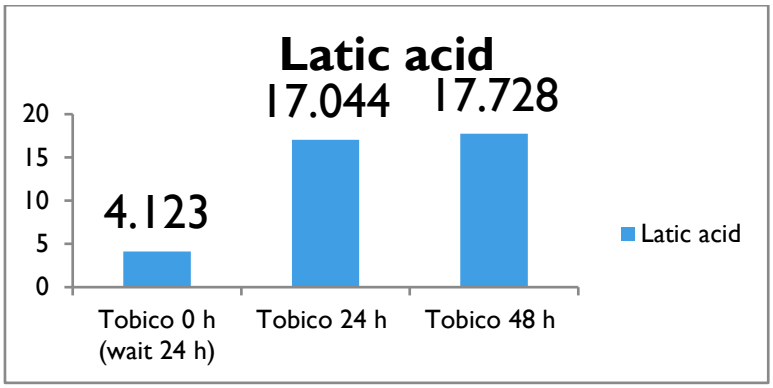
Ion liquid chromatography using an IonPac® AS11 column was applied to identify and quantify the organic acids and inorganic anions present in Tibicos fermentation broth at 0, 24, and 48 hours[12]. As shown in **Figures 69–81** and **Table 3 (Ion Content Table)**, the following key findings were observed:

Table 4. Ion and organic acid content of Tibicos fermentation broth at 0, 24, and 48 hours.

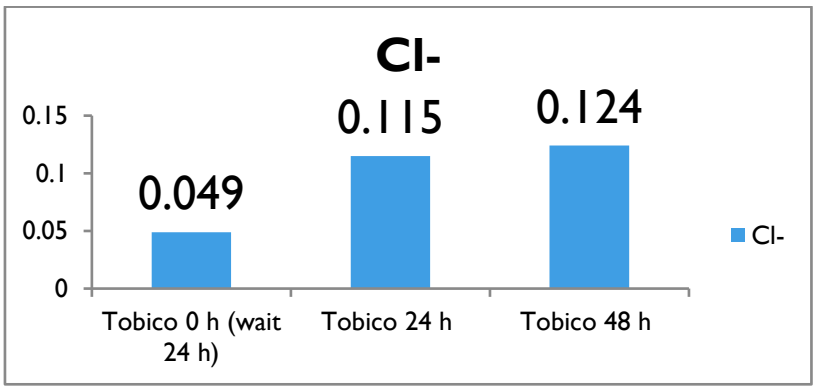
Analyte	0 hr (Brown Sugar Water)	24 hr	48 hr	Trend
Lactic acid	Present	Present	Present	Increases with fermentation time
Chloride (Cl^-)	Present	Present	Present	Increases with fermentation time
Malate	Not detected	Present	Present	Appears after fermentation; increases over time
Sulfate (SO_4^{2-})	Present	Present	Present	Relatively stable over time
Oxalic acid	Not detected	Present	Present	Appears after fermentation; relatively stable
Phosphate (PO_4^{3-})	Not detected	Present	Present	Appears after fermentation; increases over time
Citrate	Not detected	Present	Present	Appears after fermentation; increases over time

Key observations are as follows:

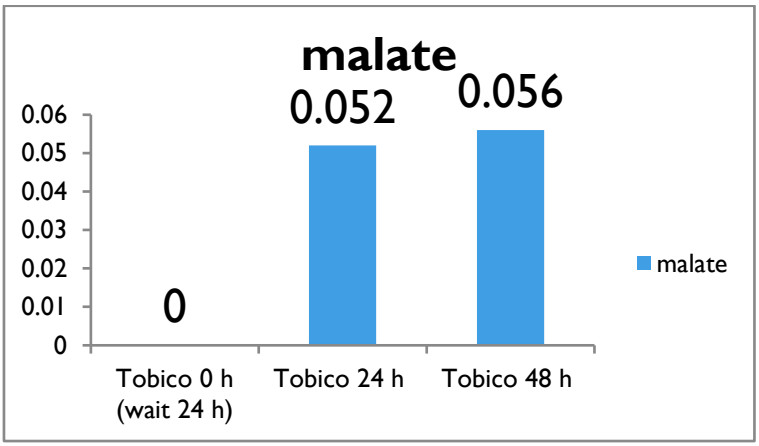
Lactic acid (**Figure 16**): Lactic acid was already detectable in the brown sugar water (0 hr control) and its concentration increased progressively with fermentation time at 24 and 48 hours.



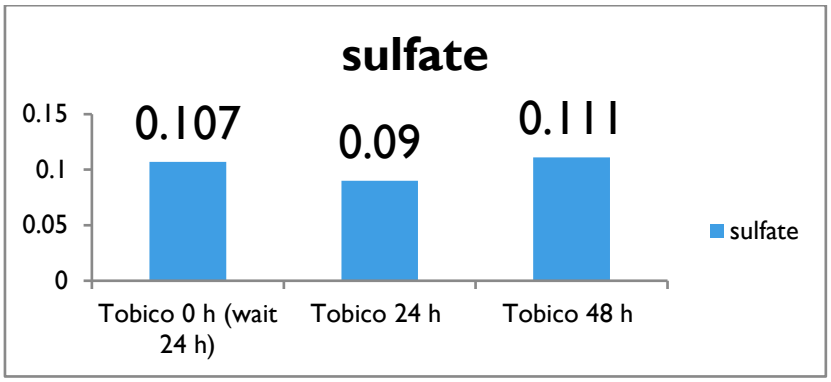
Chloride (Cl^-) (**Figure 16**): Chloride ion concentration also increased with fermentation duration.



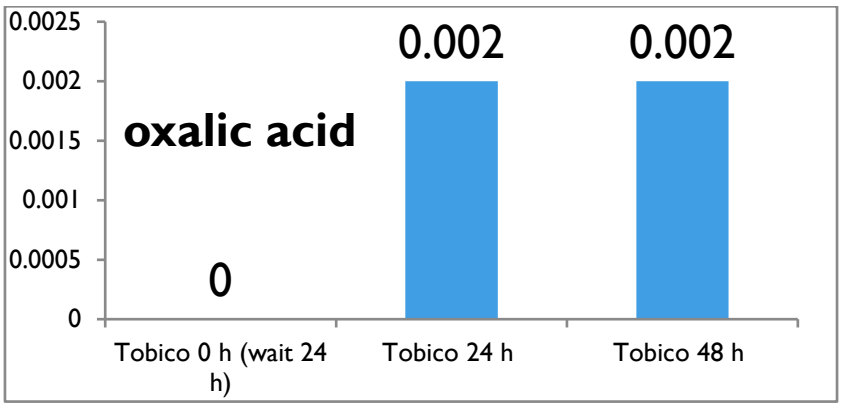
Malate (**Figure 17**): Malate was absent in the brown sugar water control and appeared only after Tibicos was added, with its concentration rising over the 24–48 hr fermentation period.



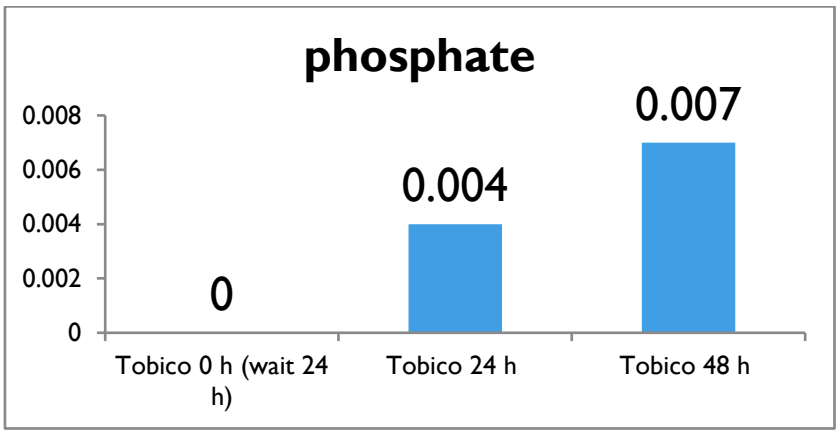
Sulfate (SO_4^{2-}) (**Figure 18**): Sulfate was detected in the brown sugar water and showed negligible change during fermentation.



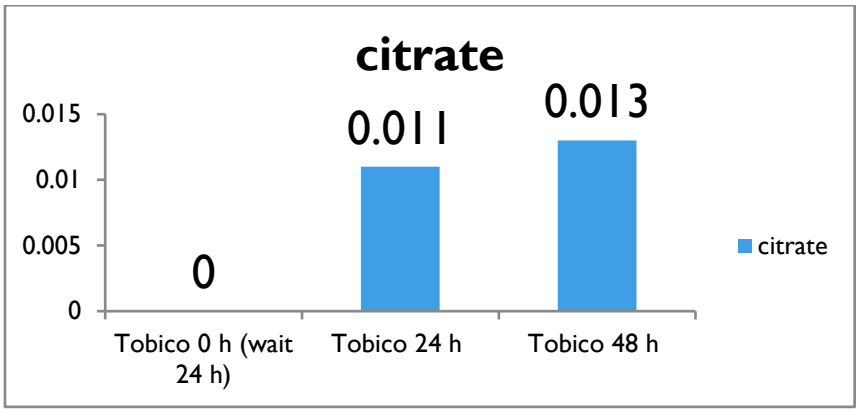
Oxalic acid (**Figure 19**): Oxalic acid was not present in the control and appeared only upon Tibicos fermentation, with a relatively stable concentration across 24–48 hr.



Phosphate (PO_4^{3-}) (**Figure 20**): Phosphate was absent in the control and emerged after fermentation, increasing progressively with time.



Citrate (**Figure 21**): Citrate was similarly absent in the control and appeared following fermentation, with its concentration increasing over time.



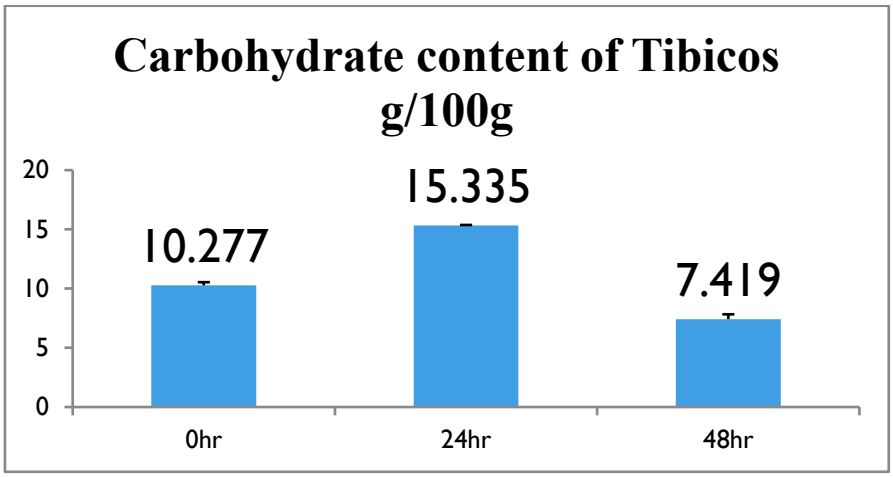
3.6 Water-Soluble Carbohydrate Content of Tibicos Fermentation Broth (0, 24, 48 hr)

The carbohydrate content of Tibicos fermentation broth was determined using the phenol-sulfuric acid method. As shown in **Figures 22** and **Tables 4–5**, carbohydrate concentration **decreased progressively** with increasing fermentation time, indicating active consumption of sugars by the microbial consortium during fermentation.

Table 5. OD_{492} absorbance values for carbohydrate content determination in Tibicos fermentation broth.

Time (hr)	OD_{492}	Carbohydrate Content (g/100 g)
0	Highest	Highest
24	Intermediate	Intermediate
48	Lowest	Lowest

Figure 22. Carbohydrate content of Tibicos fermentation broth at 0, 24, and 48 hours; carbohydrate concentration decreases as fermentation time increases.



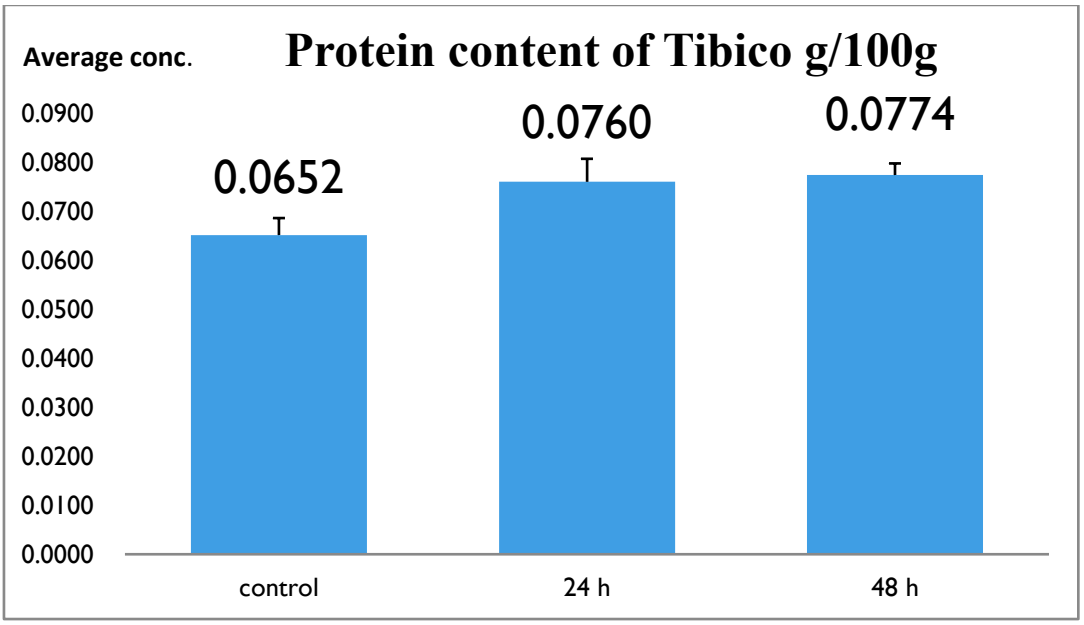
3.7 Protein Content of Tibicos Fermentation Broth (0, 24, 48 hr)

Protein content was measured using the Bradford assay. In contrast to the carbohydrate profile, protein concentration **increased progressively** with fermentation time, as shown in **Figures 23** and **Tables 6**. This suggests active metabolic secretion or cell lysis of the Tibicos microbial community during fermentation[12].

Table 6. OD_{595} absorbance values for protein content determination in Tibicos fermentation broth.

Time (hr)	OD_{595}	Protein Content (mg/mL)
0	Lowest	Lowest
24	Intermediate	Intermediate
48	Highest	Highest

Figure 23. Protein content of Tibicos fermentation broth at 0, 24, and 48 hours; protein concentration increases as fermentation time increases.

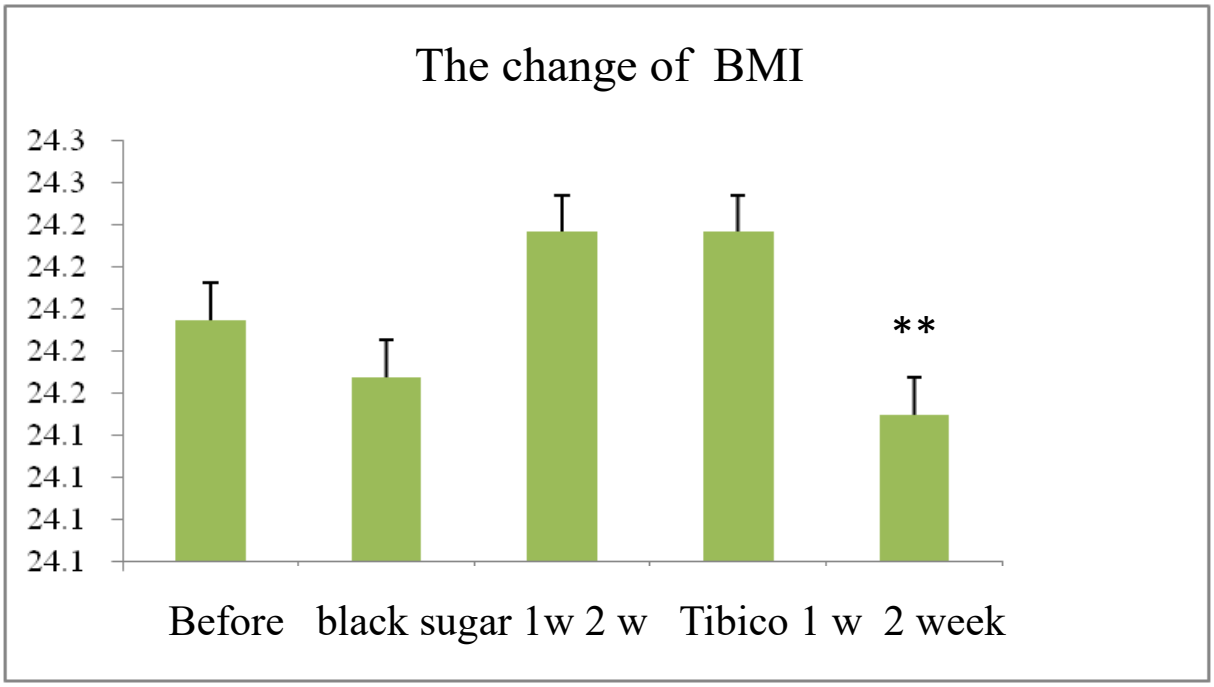


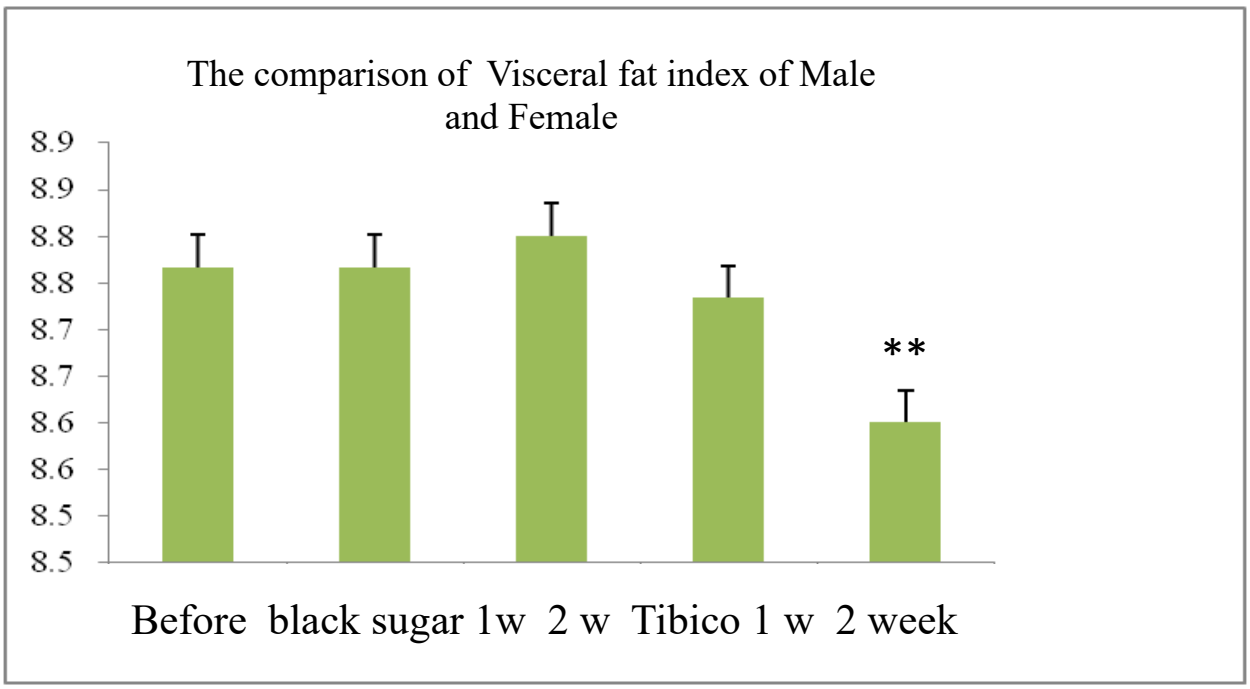
3.7 Human Physiological Evaluation

In the pilot study involving 30 subjects (age >45), two weeks of Tibicos consumption resulted in significant physiological changes compared to the brown sugar control group[5]:

1. **Visceral Fat:** There was a **statistically significant reduction in the visceral fat index ($P < 0.05$)** for the total group.
2. **Male Subgroup Analysis:** For male subjects, the reduction in weight ($P = 0.013$) and BMI ($P = 0.014$) reached statistical significance. Those with high initial visceral fat saw the most pronounced improvements ($P < 0.01$)
3. **Blood Pressure:** While physical measurements showed varied trends, subjects reported a subjective sense of circulatory stability.

Figure 24: Comparison of Body Metrics Before and After Tibicos Consumption A bar chart showing the decrease in Weight (kg), BMI, and Visceral Fat Index for male subjects after 2 weeks of Tibicos consumption, with significant P-values marked.





3.8. Subjective Health Improvements

Questionnaire analysis revealed that participants felt significantly better across multiple health dimensions ($P < 0.05$). The most notable improvements were reported in **sleep quality (insomnia), nasal allergies, and bowel regularity (constipation)**[5].

IV. Discussion

A. Scientific Validation of Metabolites and Health Claims

Traditional folklore has long associated Tibicos with metabolic and circulatory benefits, yet scientific evidence has remained sparse. This integrated study provides a chemical basis for these claims. Our HPLC analysis revealed a significant increase in lactic acid (reaching 17.728 mg/mL), which is well-documented to enhance digestive health and regulate the gastrointestinal environment.

Furthermore, the detection of citrate (0.013 mg/mL) is particularly relevant to the observed reduction in visceral fat, as citric acid is known to inhibit fat accumulation and facilitate metabolic mechanisms. The presence of malate (0.056 mg/mL) aligns with subjects' reports of blood pressure stability, given its known role in improving hypertensive conditions and activating pulmonary arterial function[17].

B. Microbial Symbiosis and Probiotic Potential

The identification of six Bacillus strains and three yeast strains highlights the complexity of the Tibicos symbiotic community[18, 19]. Of particular clinical interest is the presence of Bacillus circulans, which has been identified in literature as a source of fibrinolytic enzymes that help prevent thrombosis-related diseases. This provides a potential mechanism for the subjective improvements reported by subjects regarding circulatory comfort and reduced dizziness.

Additionally, the yeast Aureobasidium pullulans is recognized for its high survival rate and potential as a valuable probiotic strain[20, 21]. These findings suggest that Tibicos is not merely a sugar-fermented drink but a bio-active probiotic delivery system.

C. Physiological Impact and Subjective Improvements

The pilot human study demonstrated that consistent consumption of Tibicos broth leads to measurable health outcomes. The statistically significant reduction in visceral fat ($P < 0.05$) for the whole group, and specifically for males with high baseline levels ($P < 0.01$), confirms the beverage's potential in managing metabolic syndrome. While physical weight and BMI did not reach significance for the total group, the significant downward trend in male subjects ($P < 0.05$) suggests that Tibicos may be a useful adjunct in weight management for specific demographics. Subjective data from Likert-scale questionnaires revealed highly significant perceived improvements in insomnia, nasal allergies, and bowel regularity. It is worth noting that a "placebo effect" was observed during the initial two-week control phase with pure brown sugar water, where some subjects reported minor comfort. However, the scores significantly increased following the switch to Tibicos fermentation broth, particularly in dimensions of neurological and gastrointestinal comfort.

D. Challenges in Standardization and Future Directions

Despite the positive results, some physiological markers such as blood pressure and body age did not achieve statistical significance across the entire sample. This lack of significance may be attributed to the short duration of the intervention (2 weeks) and the relatively small sample size. To achieve more robust clinical data, future studies should implement a longer intervention period and include a "washout" phase (intestinal emptying period) between the control and experimental groups to eliminate carry-over effects from residual sucrose metabolism.

Furthermore, because Tibicos growth and metabolite production are highly sensitive to environmental factors as evidenced by the 45% weight gain in our controlled 25°C environment. The development of a standardized industrial cultivation protocol is essential. Future research should also incorporate a wider array of metabolic markers, such as fasting blood glucose, cholesterol, and triglycerides, to fully capture the beverage's impact on metabolic syndrome

V. Conclusion

This study successfully established a comprehensive scientific profile for Tibicos (Water Kefir), bridging the gap between traditional folklore and modern evidence-based medicine[5]. Through a combination of molecular identification, chemical metabolite profiling, and a pilot human trial[12], we have demonstrated that Tibicos fermentation broth is a potent source of bio-active compounds with measurable health benefits.

Our microbial analysis confirmed a diverse symbiotic community dominated by the Bacillus genus (including B. circulans and B. oceanisediminis) and specific yeast strains such as Aureobasidium pullulans. These microbes facilitate a dynamic fermentation process that significantly enhances the broth's nutritional profile, resulting in a 45% increase in grain biomass and a substantial rise in beneficial organic acids over 48 hours. Specifically, the marked increase in lactic acid (reaching 17.728 mg/mL), malate, and citrate explains the beverage's efficacy in aiding digestion, regulating blood pressure, and inhibiting fat accumulation.

The human pilot study involving 30 subjects provided the first clinical evidence for Tibicos as a functional drink in a local context. The statistically significant reduction in the visceral fat index ($P < 0.05$) and the targeted improvements in BMI and weight for male subjects underscore its potential in managing metabolic syndrome.

Furthermore, highly significant subjective improvements in sleep quality, bowel regularity, and respiratory comfort validate the beverage's holistic impact on physical well-being.

In conclusion, Tibicos fermentation broth represents a safe, non-dairy, and effective pilot model for functional beverage development. Its ability to deliver high concentrations of probiotics and metabolic-regulating

organic acids makes it an ideal candidate for large-scale production aimed at aging populations and health-conscious consumers.

Future research should prioritize the establishment of standardized industrial cultivation protocols and explore long-term clinical interventions to fully unlock the therapeutic potential of this traditional tonic for global public health.

References

[1] G. Diosma, D. E. Romanin, M. F. Rey-Burusco, A. Londero, and G. L. Garrote, "Yeasts from kefir grains: isolation, identification, and probiotic characterization," *World journal of microbiology & biotechnology,* vol. 30, no. 1, pp. 43-53, Jan 2014, doi: 10.1007/s11274-013-1419-9.

[2] M. Pidoux, M. F. Pilet, and V. Ripoche, "Growth performances of Lactobacillus hilgardii immobilized in dextran gel and in continuous fermentation " *World Journal of Microbiology and Biotechnology,* vol. 8, no. 4, pp. 393-398, 1992, doi: 10.1007/bf01198752.

[3] C. Garofalo *et al.*, "Bacteria and yeast microbiota in milk kefir grains from different Italian regions," *Food Microbiology,* vol. 49, no. 0, pp. 123-133, 8// 2015, doi: http://dx.doi.org/10.1016/j.fm.2015.01.017.

[4] B. Verde. "Tibicos." https://buhayverde.wordpress.com/2012/03/16/tibicos/ (accessed.

[5] T.-C. Hsu, "The Pilot Study of Tibicos as a Model to Develop Healthy Drinks," *Master Thesis of Department of Biological Science & Technology, I-Shou University,* vol. 2012, no. 1, pp. 1-144, 2012.

[6] C. Puerari, K. T. Magalhães, and R. F. Schwan, "New cocoa pulp-based kefir beverages: Microbiological, chemical composition and sensory analysis," *Food Research International,* vol. 48, no. 2, pp. 634-640, 10// 2012, doi: http://dx.doi.org/10.1016/j.foodres.2012.06.005.

[7] M. R. Corbo, A. Bevilacqua, L. Petruzzi, F. P. Casanova, and M. Sinigaglia, "Functional beverages: the emerging side of functional foods: commercial trends, research, and health implications," *Comprehensive reviews in food science and food safety,* vol. 13, no. 6, pp. 1192-1206, 2014.

[8] S.-P. Tu, "The study of Tibicos fermentation product as pilot model of healthy drinks " *Master thesis, Department of Biological Science & Technology, I-Shou University.,* vol. 1, pp. 1-120, 2015.

[9] C. Schabereiter-Gurtner *et al.*, "16S rDNA-based identification of bacteria from conjunctival swabs by PCR and DGGE fingerprinting," *Investigative ophthalmology & visual science,* vol. 42, no. 6, pp. 1164-1171, 2001.

[10] M. Oros-Sichler, N. C. Gomes, G. Neuber, and K. Smalla, "A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities," *Journal of Microbiological Methods,* vol. 65, no. 1, pp. 63-75, 2006.

[11] R. B. Moyes, J. Reynolds, and D. P. Breakwell, "Differential staining of bacteria: gram stain," *Current Protocols in Microbiology,* vol. 15, no. 1, pp. A. 3C. 1-A. 3C. 8, 2009.

[12] L. Hsiao-Han, B.-S. Tu, Y.-L. Chen, and W. Heng-Long, "The Study of Tibicos Fermentation Product as Pilot Model of Healthy Drinks," *Proceedings of Engineering and Technology Innovation,* vol. 8, p. 23, 2018.

[13] N. J. Kruger, "The Bradford method for protein quantitation," *The protein protocols handbook,* pp. 17-24, 2009.

[14] W. Chen, L. Gao, L. Song, M. Sommerfeld, and Q. Hu, "An improved phenol-sulfuric acid method for the quantitative measurement of total carbohydrates in algal biomass," *Algal Research,* vol. 70, p. 102986, 2023.

[15] S. C. Izah, L. Sylva, and M. Hait, "Cronbach's alpha: A cornerstone in ensuring reliability and validity in environmental health assessment," *ES Energy and Environment,* vol. 23, p. 1057, 2023.

[16] Z. Zhang, T. Sangsawang, K. Vipahasna, and M. Pigultong, "A mixed-methods data approach integrating importance-performance analysis (IPA) and Kaiser-Meyer-Olkin (KMO) in applied talent cultivation," *Journal of Applied Data Sciences,* vol. 5, no. 1, pp. 256-267, 2024.

[17] H. E. Romero-Luna *et al.*, "Probiotic potential of Lactobacillus paracasei CT12 isolated from water kefir grains (Tibicos)," *Current Microbiology,* vol. 77, no. 10, pp. 2584-2592, 2020.

[18] M. G. da CP Miguel, P. G. Cardoso, K. T. Magalhães, and R. F. Schwan, "Profile of microbial communities present in tibico (sugary kefir) grains from different Brazilian States," *World Journal of Microbiology and Biotechnology,* vol. 27, no. 8, pp. 1875-1884, 2011.

[19] B.-A. Mohammadou, G. Le Blay, C. M. Mbofung, and G. Barbier, "Antimicrobial activities, toxinogenic potential and sensitivity to antibiotics of Bacillus strains isolated from Mbuja, an Hibiscus sabdariffa fermented seeds from Cameroon," *African Journal of Biotechnology,* vol. 13, no. 35, p. 3617, 2014.

[20] H. E. Romero-Luna, H. Hernández-Sánchez, R. M. Ribas-Aparicio, P. I. Cauich-Sánchez, and G. Dávila-Ortiz, "Evaluation of the probiotic potential of Saccharomyces cerevisiae strain (C41) isolated from Tibicos by in vitro studies," *Probiotics and antimicrobial proteins,* vol. 11, no. 3, pp. 794-800, 2019.

[21] S. Prasongsuk, P. Lotrakul, I. Ali, W. Bankeeree, and H. Punnapayak, "The current status of Aureobasidium pullulans in biotechnology," *Folia microbiologica,* vol. 63, no. 2, pp. 129-140, 2018.