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Original Research Article

Molecular Characterization of Microbial Community Involved in the Production of Baobab (*Adansonia digitata*) Pulp Yoghurt

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Abstract: This study investigated the microbial dynamics, diversity, and succession patterns of yoghurt enriched with baobab (*Adansonia digitata*) fruit pulp during a 15-hour fermentation period. Baobab pulp was incorporated at 10% (w/v) into pasteurized milk, and fermentation was carried out at 45°C. Microbial analyses included serial dilution, cultural characterization, biochemical tests, and molecular identification using 16S and 18S rRNA sequencing. Results showed a progressive and significant ($p < 0.05$) increase in microbial populations over time. Total bacterial counts rose from 1.2×10^2 CFU/mL at 0 hours to 6.00×10^5 CFU/mL at 15 hours, while Lactobacillus populations increased from 0 to 5.62×10^5 CFU/mL. Fungal counts also increased significantly from 0 to 1.32×10^4 CFU/mL across the fermentation period. The predominant beneficial bacteria identified were *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, and *Leuconostoc* sp., confirming active lactic acid fermentation. Fungal isolates included *Aspergillus niger*, *Mucor* sp., *Botrytis cinerea*, and *Penicillium maximae*. The detection of *Staphylococcus aureus* suggests possible contamination, emphasizing the need for strict hygiene during processing. Overall, the findings demonstrate that baobab-enriched yoghurt supports robust fermentative microbial activity and contains diverse microbial communities, while highlighting the importance of aseptic production conditions to ensure product safety and quality.

Keywords: Fermentation, Microbial Populations, Yoghurt, Baobab.

INTRODUCTION

The baobab tree (*Adansonia digitata*) is endemic to the African continent, where it has significant cultural, nutritional, and economic value. It is often referred to as the "Tree of Life" due to its ability to provide essential resources such as food, water, and shelter in the harshest of climates (Maptouom *et al.*, 2020). The baobab tree's resilience in arid and semi-arid regions makes it an invaluable asset for communities that face frequent droughts and food insecurity (FAO, 2020). The tree is characterized by its massive trunk, which can store up to 120,000 liters of water, enabling it to survive prolonged dry periods (Adams *et al.*, 2019). Baobab trees produce large, gourd-like fruits that contain a dry, white pulp. This pulp is exceptionally nutrient-dense, providing a rich source of vitamin C, antioxidants, calcium, magnesium, potassium, and dietary fiber (Olatoye *et al.*, 2021).

The nutritional profile of baobab pulp has garnered interest from the global health community, particularly for its potential to combat malnutrition in developing countries. Studies have shown that baobab fruit pulp contains ten times the vitamin C of oranges, making it one of the richest sources of this essential vitamin (Singh *et al.*, 2021). In traditional African medicine, the baobab fruit pulp is used to treat various ailments, including dysentery, malaria, smallpox, and gastrointestinal disorders. The pulp's high antioxidant

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content helps reduce inflammation and supports immune function (Ogundare *et al.,* 2021). Additionally, the seeds, leaves, and bark of the baobab tree have medicinal uses, with the seeds being used as an anti-inflammatory agent and the leaves being consumed for their antipyretic properties (Iruene *et al.,* 2021).

Yoghurt, a fermented dairy product, is widely consumed for its probiotic properties and nutritional value. Combining baobab pulp with yoghurt can enhance the product's nutritional profile by adding vitamins, minerals, and antioxidants, making it a functional food with numerous health benefits (Momanyi *et al.,* 2020). The fermentation process involved in yoghurt production is carried out by lactic acid bacteria (LAB), primarily *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. These microorganisms ferment lactose, the sugar in milk, into lactic acid, which acts as a preservative by lowering the pH and inhibiting the growth of spoilage organisms (Iruene *et al.,* 2021). The LAB used in yoghurt production also contribute to the product's health benefits by improving gut health, enhancing immune function, and providing relief from lactose intolerance (Kaimba *et al.,* 2020).

MATERIAL AND METHODS

Sample Collection and Preparation

Baobab fruits were collected from mature baobab trees located in the arid regions of northern Nigeria. The fruits were carefully selected based on their size, color, and absence of physical damage. Once collected, the fruits were transported to the laboratory for further processing. In the laboratory, the fruits were washed thoroughly with clean water to remove any dirt or contaminants. The hard outer shells of the baobab fruits were cracked open using a mallet, and the pulp was extracted. The pulp was then separated from the seeds and fibers using a clean, sterile sieve. The sieved pulp was collected in sterile containers and stored at -20°C until further use as previously described by Mounjouenpou *et al.* (2018).

Figure 1: Baobab Pulp Fruits (Ogundare *et al.,* 2021)

Yoghurt Production

The baobab pulp was used as an ingredient in the yoghurt production process as previously described by Adelekan *et al.,* (2020) and Stadlmayr *et al.,* (2020) with little modification. Fresh cow milk was obtained from a local dairy farm and was pasteurized by heating it to 85°C for 15 minutes to eliminate any pathogenic microorganisms. After pasteurization, the milk was cooled to 45°C, the optimal temperature for the growth of yoghurt starter cultures. Starter cultures containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were added to the cooled milk. The inoculated milk was then divided into two batches. In the first batch, 10% (w/v) of baobab pulp was added to the milk, while the second batch served as the control without any baobab pulp. Both batches were incubated at 45°C for 6 hours to allow fermentation to occur.

Microbial Analysis

Serial Dilution

A portion of 10 ml of the sample was added into a flask containing 90 ml of distilled water and mixed carefully. A portion of 1 ml of the homogenate was transferred into a test tube containing 9 ml of the diluent. It was mixed carefully with a fresh pipette by aspirating 10 times, then from the second dilution, another 1 ml was transferred into the next tube containing 9 ml of the sterile diluent. This process was repeated with the same steps using the 1st, 2nd, 3rd, 4th, and 5th tubes of the diluent. Microbial analysis was conducted to determine the microbial diversity and load in the baobab pulp yoghurt as previously described by Dauda *et al.,* (2020). Serial dilutions of the yoghurt samples were prepared using sterile saline solution.

Determination of Bacterial and Fungal Diversity

A portion of 1 ml of the sample dilution was pipetted from the 1st, 2nd, 3rd, 4th, and 5th tubes into each of the appropriately marked sterile petri dishes followed with a 15mls of molten nutrient agar (NA) and potatoes dextrose agar (PDA) to a dedicated Petri-plates. The plates containing NA were incubated at 37^{O}C for 24 hours while those with PDA were incubated at room temperature (25°C) for 25 to 14 days for bacteria and fungi. Colonies that appeared on the plates were counted, and the results were expressed as colony-forming units per gram (CFU/g) of yoghurt (Adelekan *et al.,* 2020).

Isolation and identification of Bacteria

Aliquots from each dilution were plated on different selective and differential media to isolate and identify various microorganisms as previously described by Dauda *et al.,* (2020). The media used included De Man, Rogosa, and Sharpe (MRS) agar for lactic acid bacteria, and Soy-casein Digest AGAR (SCDA). The plates were incubated at 37^{O}C for 18 to 48 hours. Representative colonies were selected based on their morphological characteristics and were further subjected to Gram staining biochemical tests (indole, VP, methylred, citrate utilization, catalase, coagulase and urease test) and molecular detection for identification purposed (Iruene *et al.,* 2021).

Isolation and Identification of Fungi

For the isolation of yeast and mold, the pour plate technique was used as previously described by Adelekan and Saleh, (2020). A portion of 1 ml of the sample dilution was pipetted from the 1st, 2nd, 3rd, 4th, and 5th tubes into each of the appropriately marked sterile petri dishes. The plates were incubated at room temperature (25°C) for 5 to 14 days (Eze *et al.,* 2014). Representative colonies were selected based on their morphological characteristics, microscopic examination and molecular detection for identification as described by described by Větrovský *et al.,* (2020).

Monitoring of Microbial and Chemical Changes

The microbial and chemical changes in the baobab fruit pulp yogurt were monitored through periodic pH measurements to track pH decrease at three-hour intervals, temperature measurements to note increases or decreases at three-hour intervals, and determination of total acidity (lactic acid) at three-hour intervals. Other methods used to monitor the changes included microbial analysis, proximate analysis every three hours, mineral determination every three hours during the fermentation process, and measurement of enzyme activity every three hours (Eze *et al.,* 2014; and Titilayo and Musliu, 2016; Mwangi, 2023).

Molecular Analysis

Fungal DNA Extraction

The method adopted by Větrovský *et al.,* (2020) was used for the extraction of DNA from fungi. Fungal mycelium was produced in 20 ml of Potato Dextrose Broth. The mycelium was harvested by filtration through mesh sieves, washed with sterile water, and deposited on Whatman filter paper to remove excess water. The mycelium was then ground to a fine powder in liquid nitrogen. DNA was extracted using a nitrogen-based DNA extraction kit (Dongsheng Biotech) following the manufacturer's instructions.

PCR Amplification

The PCR amplification method adopted by Větrovský *et al.* (2020) was used. PCR amplification was carried out in a total volume of 25 µl, containing 20 mg of genomic DNA, 1X PCR buffer (20 mM Tris-HCL, 10 mM (NH4)2SO4, 10 mM $MgSO_4$, 0.1% Triton X-100), 0.2 units of Taq DNA polymerase, and ITS1/ITS4 primers. ITS1 and ITS4 are universal fungal primers, with ITS1 serving as the reverse primer. Restriction Fragment Length Polymorphism (RFLP) was used, with the forward primer ITS4 having a base pair sequence of 5-TCCTCCGCTTGATATGC-3. ITS is a standard marker for fungal DNA barcoding. The PCR amplification followed this temperature protocol: an initial step of 2 minutes at 94°C, 90 seconds at 52°C, 2 minutes at 72°C, and a final step of 7 minutes at 72°C. Electrophoresis of PCR-amplified products was performed in 1.5% agarose gel. The PCR products were stained with ethidium bromide and visualized with 305 nm ultraviolet light.

Bacterial DNA Extraction and Amplication

The method adopted by Mwangi *et al.,* (2023) was used for DNA extraction from each bacterial isolate, utilizing the standard phenol/chloroform method. The quality of the DNA was checked by electrophoresis in 0.8% agarose gel and quantified using a Nano-Drop ND-1000 spectrometer (Eppendorf, Germany). The PCR amplification of the 16S rRNA gene from isolated DNA was performed using a universal oligonucleotide primer pair 27F (5 AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-TACGGTTACCTTGTTACGACTT-3) (Lane, 1991) in a thermal cycler (Thermo Fisher, United States). The reaction mixture, conditions, and protocol for the polymerase chain reaction amplification were as follows: a final volume of 50 µl containing Go Taq Green Master Mix (2x) (M7122, Promega, United States), 10 µM of the forward primer, 10 µM of the reverse primer, and nuclease-free water (NEB). The PCR conditions were set to 94°C for 10 minutes, followed by 35 cycles of 94°C for 1 minute, 65°C for 1 minute, and 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. The PCR product was detected by electrophoresis using 1% agarose, and the bands were stained with 7 µl/100 ml of ethidium bromide and visualized using a Gel Doc EZ Imager (Bio-Rad, United States). A standard 100-base pair DNA ladder was used for verifying the amplicon size. The amplified PCR products were purified using PEG (polyethylene glycol)-NaCl (sodium chloride) (20% w/v of PEG, 2.5 M NaCl).

16S rRNA Gene Sequencing

The method adopted by Mwangi *et al.,* (2023) was used for 16S rRNA gene sequencing. PCR products were set up in 5 µl volumes for single-primer amplification using the same universal primers 27F (5-AGAGTTTGATCCTGGCTAG-3) and 1492R (5-TACGGTTACCTTGTTACGACTT-3) for separate reactions of each primer. The PCR reaction conditions were: denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and elongation at 60°C for 2 minutes, followed by a stop reaction at 4°C. The amplicons were then precipitated with 1 µl sodium acetate (3 M, pH 5.2) and 24 µl of absolute alcohol, mixed briefly in a vortex, incubated at room temperature for 15 minutes,

centrifuged at 12,000 rpm for 20 minutes, washed with 70% ethanol, dried, and suspended in 10 µl formamide. Sequencing of the amplicons was performed using the Sanger sequencing method.

Statistical Analysis

The data obtained from the various analyses were subjected to statistical analysis using Statistical Package for the Social Sciences (SPSS) software. Descriptive statistics, including mean and standard deviation, were calculated for each parameter. Analysis of variance (ANOVA) was performed to determine the significance of differences between the baobab pulp yoghurt and the control. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Microbial Counts of Yoghurt Supplemented with Baobab Pulp

During the 15-hour fermentation of yoghurt supplemented with baobab fruit pulp, significant ($p<0.05$) microbial growth was observed and shown in Table 1. The bacterial count increased from 1.2×10^2 CFU/mL at 0 hour fermentation to 6.00×10^5 CFU/ml at 15 hour fermentation, reflecting active fermentation. Similarly, the fungal count show significant increase 0.00 to 1.32×10^4 CFU/ml at 0 and 15 hour fermentation, respectively. In the same vein, *Lactobacillus* spp also shows similar trend with significant increase ($p<0.05$) from 0 to 5.62×10^5 CFU/ml.

Table 1: Effect of fermentation time on bacterial and fungal counts of yoghurt supplemented with baobab pulp

		Parameters (CFU/mL)		
S/N	**Fermentation time (hours)**	**Lactobacillus count**	**Bacterial count**	**Fungal count**
1.	0	0.00^{f}	$1.2^{f} \times 10^2$	0.00^{f}
2.	3	$3.31^{e} \times 10^4$	$6.8^{e} \times 10^4$	$2.01^{e} \times 10^3$
3.	6	$1.10^{d} \times 10^5$	$1.16^{d} \times 10^5$	$6.02^{d} \times 10^3$
4.	9	$2.02^{c} \times 10^5$	$2.16^{c} \times 10^5$	$1.01^{c} \times 10^4$
5.	12	$2.31^{b} \times 10^5$	$2.56^{b} \times 10^5$	$1.21^{b} \times 10^4$
6.	15	$5.62^{a} \times 10^5$	$6.00^{a} \times 10^5$	$1.32^{a} \times 10^4$

Key Words, CFU/ml – Colony Forming Unit per mills.

Figure in the same column with the same superscript are not significantly different at ($P<0.05$)

Microbial Diversity in the Yoghurt Supplemented with Baobab Fruit Pulp

The microbial diversity in the yoghurt supplemented with baobab fruit pulp is shown in tables 2, 3 and 4 included several beneficial bacteria and fungi. The bacteria identified and characterized were *Lactobacillus delbrueckii subsp. bulgaricus strain 37-2, Leuconostoc sp. strain AceX2, Streptococcus thermophilus strain HBD5044*and *Staphylococcus aureus strain Ng016* with *Lactobacillus delbrueckii subsp. bulgaricus strain 37-2,* playing a dominant role in fermentation. The fungi isolated included *Aspergillus niger*, *Mucor sp.P20-S2-1*, *Botrytis cinera* isolate Al3 and *Penicilliummaximae strain ZJ11B02*.

Table 2: Phenotypic characterization of bacterial isolates of yoghurt supplemented with baobab pulp

Biochemical									
Morphology	**Microscopy**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Isolates**
Colonies are small, round, smooth, and opaque, occurs singly or in short chains	+	+	-	+	-	-	-	-	*Lactobacillus delbrueckii subsp. bulgaricus strain 37-2*
Colonies are small, round, smooth, and translucent, occurs in pairs or short chains	+	+	-	+	+	-	-	-	*Leuconostoc sp. strain AceX2*
Colonies are large, round, smooth, and opaque, producing a golden-yellow pigment, occurs in clusters.	+	+	-	+	+	+	+	+	*Staphylococcus aureus strain Ng016*
Colonies are small, round, smooth, and translucent, with no pigmentation, occur in chains	+	+	-	-	-	-	-	-	*Streptococcus thermophilus strain HBD5044*

Key words; 1 – Indole test, 2 – Methyl red test, 3 - Voges-Proskauer test, 4 – Citrate utilization test, 5 – Catalase test, 6 – Urease test, 7 – Coagulase Test

Table 3: Cultural and microscopic morphological characteristics of the fungal isolates of yoghurt supplemented with baobab pulp

Cultural Characteristics	Microscopic Characteristics	Isolates
Blackish colonies on SDA or pin like black growth	Non-branched conidiophores with bulb end carries conidia like sunrays	*Aspergillusniger.*
Grayish-white mycelium with a characteristic "botryose" (clustered) appearance.	Globoseseptateelliptical conidia on conidiophores	*Botrytis cinerea isolate AI3*
Cotton like white growth spotted with black color	Sporangia contain spores, do not have rhizoids	*Mucor sp. P20-S2-1*
Green or green greyish color colonies growth over fruits especially citrus	Brush-like conidiophore carries conidia	*Penicilliummaximae strain ZJ11B02*

Table 4: Summary of 16s and 18sr RNA Sequencing of the Isolates to Identify the Closest Homologs

Sample ID	Sequence bp	Identity of the closest homologs of the sequence	% identity	Accession Nº of the closest homolog
S5	936	*Lactobacillus delbrueckii subsp. bulgaricus* strain 37-2	99.79%	MG437366.1
S4	511	*Leuconostoc* sp. strain AceX2	96.42%	OQ994734.1
S2	265	*Staphylococcus aureus strain Ng016*	97.87%	MH517387.1
S3	1169	*Streptococcus thermophilus* strain HBD5044	97.22%	MW725290.1
S8	500	*Aspergillusniger*	99.59%	AB030916.1
S6	472	*Botrytis cinerea* isolate AI3	95.51%	PP761243.1
S9	519	*Penicilliummaximae* strain ZJ11B02	98.45%	PP385644.1
S7	746	*Mucor*sp. *P20-S2-1*	98.12%	LC769414.1

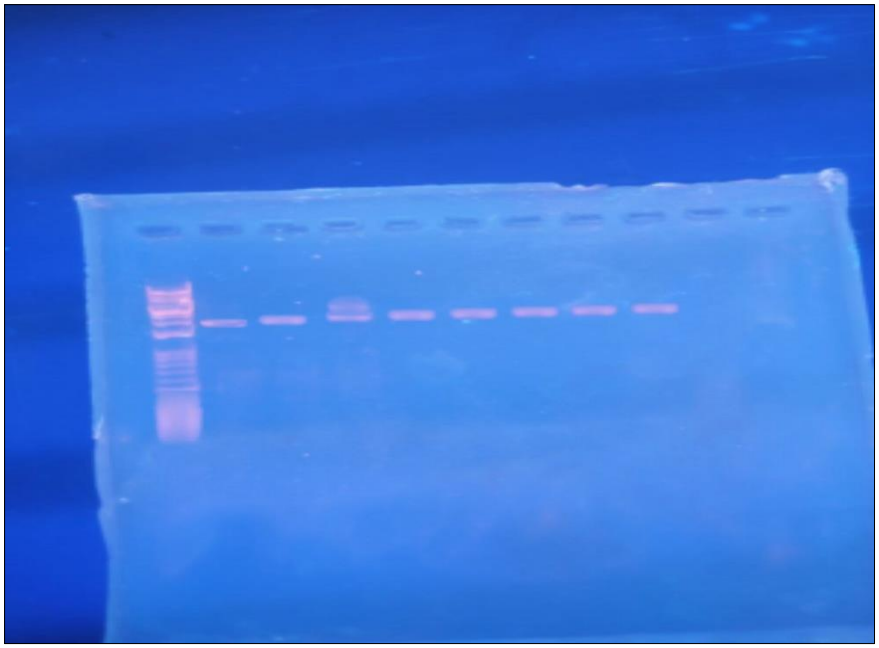

Figure 1: Gel Electrophoresis of the amplified 16s rRNA genes

DISCUSSIONS

Yogurt is a fermented dairy product that is produced by acidifying milk with certain yogurt bacteria (*Lactobacillus delbruecckii* subsspecie *bulgaricus* and *Streptococcus salivarius* subspecie *thermophilus*) (Jakubowska& Karamucki, 2019). Yoghurt is rich in nutritional content and serves as a medium for microbial growth which leads to contamination. Bacteria, moulds and yeasts are the major contaminants in yoghurt (Zumunta *et al*., 2020). The result of the microbial counts of the baobab fruit pulp yoghurt in this study, comparising of both bacteria and fungi, (Table 1) reveal that the higest bacterial counts (6.0 x10^5CFU/ml) was recorded at 15hours fermentation time, while the lowest bacterial count of (1.2 x10^2CFU/ml) was recorded at 0 hours. The effect of fermentation on the bacterial counts in food products have been reported by different authors. Example, Achi *et al.,* (2007) researched on microbiological and chemical changes during fermentation of crabs for ogiri-nsiko production.

Zumunta *et al.*, (2020) reported a bacterial count of 1.4 x 10^5CFU/ml in yoghurt supplemented with baobab fruit at 3 hours fermentation which is lower than the count observed in the present study at the same fermentation time. Interestingly, the fermentation time is similar with slightly different in the formulation between the milk and the fruit pulp; higher proportion of fruit pulp may be attributed to the higher count, fruit pulp is rich in ascorbic acid and is reported to posses antibacterial property. This agreed with finding of Zumunta *et al.*, 2020 who reported that the low microbial counts of the yoghurt supplemented with fruit pulp was attributed to the antimicrobial effect of the pulp. The lactic acid bacterial count from the results show the highest counts (5.62 x10^5 CFU/ml) was obtained at 15hours fermentation time, while the least counts (3.31 x10^4CFU/ml) was obtained 3hours fermentation time it was observed that, like the previous bacterial counts, lactic acid bacterial count increases with increase in the fermentation time (0hours 0, 3hours 3.31 x10^4, 6hours 1.10 x10^5, 9hours 2.02 x10^5, 12hours 2.31 x10^5, 15hours 5.62 x10^5). The results also show that there was increase in the bacterial counts with corresponding increase in the fermentation time (0hours 1.2 x10^2, 3hours 6.8 x10^4, 6hours 1.16 x10^5, 9hours 2.16 x10^5, 12hours 2.56 x10^5 and 15hours 6.00 x10^5).

From the result of the study, species of bacteria isolated include *Lactobacillus delbrueckii subsp. bulgaricus strain 37-2, Leuconostoc sp. strain AceX2, Streptococcus thermophilus strain HBD5044;* in addition to this three lactic acid fermenters, *Staphylococcus aureus strain Ng016* was also isolated from the sample (Table 2); However this might result from contamination. From the results the figure obtained for both bacterial and *lactobacillus* counts are significantly different at (P<0.05). Occurance of *Staphylococcus aureus strain Ng016* in this study agrees with the finding Aze *et al.*, (2014) who isolated diverse microorganism including pathogenic organisms like *pseudomonas aerogenosa, Salmonella* spp., *Aspergillus* Spp. from fermenting oil beans seed. The results of the fungal counts of this study reveals the highest fungal counts of (1.32 x10^4CFU/ml) and lowest counts of (2.01 x10^3CFU/ml) at 15hours and 3hours respectively. The lowest fungal counts obtained in this study is lower than the counts reported by Zumunta *et al.*, (2020) in the third hour of fermentation (2.8 x 10^4CFU/ml), However the highest fungal counts obtained in this study was high than counts reported by Zumunta *et al.*, (2020). Species of fungi isolated from the baobab pulp yoghurt sample include; *Aspergillus niger*, *Mucor sp.P20-S2-1, Botrytis cinera* isolate Al3 and *Penicilliummaximae strain ZJ11B02* (Table 3 and 4). The results of the fungal counts were significantly different at (P<0.05) in general, the bacterial counts, *Lactobacillus* counts and fungal counts were significantely different at (P<0.05).

CONCLUSION

This study determined the microbial dynamics and diversity of yoghurt enriched with baobab fruit pulp during a 15-hour fermentation period. Results indicated that time of fermentation strongly affected the proliferation and succession of microorganisms present in yoghurt samples. Bacterial counts, fungal counts, and lactic acid bacteria populations increased successively with fermentation, indicating active microbial metabolism and the appropriateness of baobab-enriched yoghurt as a medium for the growth of fermentative microbes. *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, and *Leuconostoc* spp. were the prevalent beneficial bacteria identified, confirming the expected activity of lactic acid bacteria during yoghurt fermentation.

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