

Molecular Identification of Lactic Acid Bacteria Isolated from Fermented Coconut Yoghurt through Uncontrolled Fermentation

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Abstract

This study aimed to identify and characterize Lactic Acid Bacteria obtained from uncontrolled fermentation of coconut milk using molecular biology tools. Healthy coconut fruit was aseptically processed to obtain the milk which was afterwards allowed to ferment spontaneously under laboratory condition. Using standard microbiological methods Lactic Acid Bacteria (LAB) were isolated from the fermenting milk during fermentation. The LAB isolates were subjected to presumptive tests and subsequently to molecular analyses to confirm their identities. Genomic DNA of each LAB was extracted using commercial kits, 16S rRNA gene of the extracted DNA was amplified by Polymerase Chain Reaction (PCR) and the product sequenced by Sanger method. Similarity and phylogenetic relationship of the resulting sequences with reference strains' sequences in National Centre for Biotechnology Information database were compared using BLAST. Isolated Lactic Acid Bacteria were identified as *Lactobacillus acidophilus*, *Limosilactobacillus reuteri*, *Leuconostoc mesenteroides*, and *Lactococcus lactis*, with each exhibiting 100.00% homology to 16SrRNA gene sequence of reference strains. This study demonstrates the effectiveness of the selective isolation method and highlights the potential benefits of LAB in developing a non-dairy yoghurt from coconut milk. Further research is recommended to the use of these LAB isolates as starters for controlled fermentation of coconut milk to develop various fermented foods from this process. Also, the probiotic properties and functional characteristics of the isolated LAB species could be further explored.

Keywords: Lactic Acid Bacteria, Coconut Milk, Uncontrolled Fermentation, 16S rRNA gene, Sanger Sequencing

Word Count: 224

Introduction

Lactic Acid Bacteria (LAB) are a group of Gram-positive, facultative anaerobic or microaerophilic microorganisms that play a crucial role in food fermentation. These bacteria are capable of producing lactic acid as a primary metabolic end product, which contributes to the characteristic flavour, texture, and preservation of fermented foods. The increasing demand for non-dairy yoghurt alternatives has led to the exploration of novel substrates, such as coconut milk, for LAB fermentation. Coconut milk is a rich source of nutrients, including proteins, fats, and carbohydrates, making it an attractive substrate for LAB growth and fermentation.

This study aimed to identify and characterize LAB isolated from fermented coconut yoghurt through uncontrolled fermentation using molecular biology tools. The findings of this study contribute to our understanding of the LAB species involved in coconut milk fermentation and their potential applications in the development of non-dairy yoghurt products.

Non-dairy beverages consumption has increased in the last years due to several factors such as: food allergies and restrictions, desire to limit animal-based foods due to health concerns, and vegetarian and vegan diets. Plant-based milk alternatives are a rising trend among consumers worldwide. However, research in the development of these products is necessary to overcome problems of nutrients, emulsion stability, texture, and sensory properties of coconut milk.

Studies confirm the possible positive health-promoting properties of the use of coconut milk in the diet. In turn, microbiological changes are the subject of research in the development of fermented products, and it is to investigate the effect of individual lactic acid bacteria on the formation of a bacterial flora beneficial for the human body that contributes to its proper functioning. Fermentation is mainly the domain of lactic acid bacteria by which beneficial products are obtained, including yogurt, whose properties are intended to contribute to the improvement of human health.

Uncontrolled fermentation of coconut yoghurt involves the spontaneous growth and metabolic activities of indigenous microorganisms, resulting in a complex and dynamic microbial ecosystem that influences the product's physicochemical, sensory, and microbiological properties. The application of molecular characterization techniques, including 16S rRNA gene sequencing and phylogenetic analysis, to Lactic Acid Bacteria (LAB) populations in coconut yoghurt facilitates a comprehensive understanding of the microbial

ecology and diversity underlying fermentation, thereby enabling the precise identification and exploitation of LAB strains with desirable functional and probiotic attributes.

Material and Methods

Collection of Sample

Fresh mature coconuts were the primary raw material used in this study. These were sourced from local markets and selected based on maturity and freshness. Deionized water was employed throughout the experimental procedures to ensure purity and eliminate interference from external ions. Standard laboratory media and reagents such as MRS agar. (Trajkovska et al., 2023)

Sample Preparation

The coconuts were manually dehusked, cracked, and the meat extracted. The coconut meat was washed thoroughly, grated, and blended with warm deionized water in a 1:2 weight-to-volume (w/v) ratio. The mixture was filtered through muslin cloth to yield fresh coconut milk. This milk was then pasteurized at 85°C for 30 minutes to eliminate undesirable microorganisms and deactivate native enzymes.

Inoculation and Fermentation

A portion of the coconut milk was used for microbial isolation. Indigenous lactic acid bacteria (LAB) were isolated by plating samples on MRS agar, followed by anaerobic incubation at 37°C for 48 hours. Pure colonies were subcultured, morphologically examined, and subjected to preliminary biochemical tests. These isolates were subsequently used as starter cultures to ferment fresh samples of coconut milk.

The fermentation process involved inoculating the cooled coconut milk with 2% (v/v) of the LAB culture under aseptic conditions. The inoculated samples were incubated at 42°C for 6 to 8 hours until the pH dropped to approximately 4.6, indicating sufficient acidification. For comparative analysis, cow milk samples were inoculated with the same LAB strains and fermented under identical conditions. The fermentation process was monitored by observing changes in pH, texture, and appearance. (Anumba et al., 2023)

Purification of LAB using MRS Agar and Serial Dilution Techniques

The fermented coconut milk was serially diluted in sterile saline solution (0.85% NaCl) to obtain a dilution range of 10^{-1} to 10^{-6} . 100 μ L of each dilution was plated on MRS (de Man, Rogosa, and Sharpe) agar plates, which were incubated anaerobically at 37°C for 24-48 hours. Colonies with distinct morphologies were selected and purified by repeated streaking on MRS agar plates.

Identification of Bacteria Isolated from Coconut Water

The isolates were characterized based on their cultural and biochemical characteristics (Ikpeama *et al.*, 2019). The bacteria colonies were differentiated based on their morphological characteristics by gram staining, followed by other biochemical tests such as oxidase test, Catalase test, Methyl red test, sugar fermentation, and Indole test. (Anumba *et al.*, 2023)

Gram Staining

Gram staining was done by making a smear of each bacterium isolate from 18-24hrs old culture on the clean grease-free slide. The slide was flooded with crystal violet and left for 60secs before rinsing with distilled water; Lugol's iodine was added and allowed to act for about 60secs followed by rinsing with distilled water. The stained smear was decolorized with 95% alcohol, rinsed with distilled water, and counterstained with 1% safranin for 60 seconds, after which the stain was rinsed off with distilled water. The preparation was blot-dried and examined under an oil immersion objective (Aneja, 2022).

Biochemical Tests

Biochemical tests were carried out to have a presumptive identification of the potential bacteria. Most of the methods were done according to the microbiology laboratory manual (Cappuccino & Sherman, 2021). The biochemical tests performed were useful in bacteria identification including the catalase test, oxidase test, indole test, motility test, and sugar fermentation.

Catalase Test

Catalase tests were done to determine the ability of the bacteria to degrade hydrogen peroxide by producing the enzyme catalase. A microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small amount of bacteria from 24 hours of pure culture was placed onto the microscopic slide. A drop of hydrogen peroxide (H_2O_2) diluted with 3 % distilled water was placed onto the organisms on the microscopic slide using a dropper and observed for

immediate bubble formation (Karen, 2020). The effervescence of gas with white foam indicates a positive catalase reaction while the absence of foamy formation after 60 seconds indicates a negative catalase reaction (Karen, 2020).

Oxidase Test

Oxidase tests were done to determine the presence of the enzyme-cytochrome oxidase in the bacteria. Using an inoculating loop, a well-isolated colony from pure 24-hour culture was picked and placed onto a small piece of filter paper, and a small drop of oxidase reagent was dropped onto the filter paper and observed for colour change (Shield & Cathcart, 2010).

Indole Test

Indole production was done to determine the ability of the bacteria to degrade the amino acid tryptophan by the enzyme tryptophanase. 1g of peptone powder was added to 100 ml of distilled water, then 10 ml was dispensed into 10 McCartney bottles and it was sterilized and then allowed to cool. Using a sterile technique, small amounts of experimental bacteria from a 24hour-old pure culture were inoculated into the McCartney bottle using a loop inoculation method and then the McCartney bottle was incubated for 48 hours at 37⁰C. To test for indole production 3 drops of kovac's reagent were added directly into the McCartney bottle (MacWilliams, 2009).

Methyl red Test

Two milliliter of peptone water was inoculated with the bacteria organisms and incubated at 37⁰C for 48 hours and a drop of methyl red reagent (prepared by dissolving 0.04g of methyl red powder in 100ml of ethyl alcohol) was added to the tube. The development of red colour implies that the result is positive. In isolates that do not produce acid during glucose fermentation, the methyl red changed to yellow showing a negative result (Omemu *et al.*, 2018).

Sugar Fermentation

This tests an organism's ability to ferment sugar glucose as well as its ability to convert the end product of glycolysis, pyruvic acid to a gaseous by-product. The peptone was prepared according to the manufacturers' instruction and 3 drops of phenol red were added, the broth was dispensed in 4.5ml aliquot in the test tube containing an inverted Durham tube, the tubes were

then cotton plugged and autoclaved at 121⁰C, and each sugar (glucose, lactose, mannitol, sucrose, maltose, fructose, and galactose) were dissolved in distilled water prepared separately, the sugars were autoclaved at 121⁰C for 15 min. 0.5ml of broth was then added to 4.5ml of peptone water aseptically, the appearance of yellow colour in the medium indicated positive while lack of yellow colour appearance indicated a negative result. Also, gas formation is indicated by the appearance of gas bubbles in the Durham tubes (Nwachukwu *et al.*, 2014).

Molecular Analysis and Identification

Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers. The PCR products were run on a gel and cleaned up enzymatically using the EXOSAP method. The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™TerminatorCycleSequencingKitV3.1, BRD3-100/1000) and purified (ZymoResearch, ZR-96DNA Sequencing Clean-up Kit™, Catalogue No. D4050. Daryani *et al.*, 2020. The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample, BioEdit SequenceAlignmentEditorversion7.2.5 was used to analyse the. ab1 files generated by the ABI3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI).

Sequencing and Phylogenetic Analysis

The PCR products were sequenced using a DNA sequencer (e.g., ABI 3730xl). The sequences were analyzed using BLAST and aligned with reference sequences from the GenBank database. Phylogenetic trees were constructed using neighbor-joining.

Results

The biochemical reactions observed across the 22 isolates are presented in Table 1,2and 3. Among the tests performed, the Voges-Proskauer (VP) reaction was widely positive across the isolates, indicating the production of acetoin, a neutral end product of glucose fermentation via the butylene glycol pathway. In contrast, all isolates were negative for the Methyl Red (MR) test, suggesting they do not follow the mixed-acid fermentation pathway commonly associated with enteric bacteria. The catalase test revealed that a significant number of isolates were

catalase-positive, implying their ability to detoxify hydrogen peroxide, which is characteristic of aerobic or facultatively anaerobic bacteria. Based on the biochemical profiles and microscopic observations, tentative identifications were assigned to each isolate recovered from coconut milk samples, as detailed in Table 3. This identification process was guided by key indicators such as Gram reaction, cell morphology, catalase activity, fermentation ability, motility, and other distinguishing traits. Among the first group of isolates (C1–C11), several were found to belong to genera commonly associated with lactic acid fermentation and environmental spore-formers. For example, isolate C1, a catalase-negative, Gram-positive coccus that tested positive for the Voges-Proskauer reaction, was tentatively identified as either *Leuconostoc* or *Lactococcus lactis*, both of which are known for their roles in dairy and plant-based fermentations. Isolates C2 and C3 displayed characteristics consistent with *Lactobacillus* and *Bacillus* species, respectively organisms well-recognized for their probiotic potential and ability to produce organic acids. Table 4 shows the BLAST predictions on the NCBI database. The 16S rRNA gene sequences of the LAB isolates were obtained and analyzed using BLAST. The isolates were identified as *Lactobacillus acidophilus*, *Limosilactobacillus reuteri*, *Leuconostoc mesenteroides*, and *Lactococcus lactis* based on their sequence similarity to reference strains. The phylogenetic analysis revealed that the LAB isolates clustered with reference strains. The sequences of the isolated LAB species were compared with reference strains in public databases using tools like BLAST (Basic Local Alignment Search Tool). This analysis determined the similarity between the isolates and reference strains, providing insights into the identity and phylogenetic relationships of the LAB isolates.

Table 1. Biochemical Characterizations of Isolates

PARAMETERS	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
METHYL RED	-	-	-	-	-	-	-	-	-	-	-
VOGES PROSEKAUER	+	+	+	-	+	-	+	+	-	-	+
CATALASE	-	-	+	+	+	+	+	+	+	+	
SULPHIDE	-	+	+								
INDOLE	-	-	-	-	-	-	-	-	+	-	
MOTILITY	-	-	+	+	+	+	-	-	-	-	
GLUCOSE	AG	A	A	AG	a	NC	NC	a	a		
LACTOSE	NC	A	A	A	a	NC	A	NC	NC	NC	a
CELL SHAPE	Cocci Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Cocci	Rod	
GRAM REACTION	+	+	+	-	+	-	-	-	+	+	+

Table 2 : Biochemical Characterizations of isolates (Cont.)

PARAMETERS	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22
METHYL RED	-	-	-	-	-	-	-	-	-	-	-
VOGES	-	-	-	-	-	-	-	-	-	-	-
PROSEKAUER	+	+	+	+	+	+	+	+	+	+	+
CATALASE	+	+	+	+	+	+	+	+	+	+	+
SULPHIDE	-	+	-	-	-	-	-	-	-	-	-
INDOLE	-	-	-	-	-	-	-	-	-	-	-
MOTILITY	-	-	-	-	-	-	-	-	-	-	-
GLUCOSE	-	+	+	+	+	-	-	-	-	-	-
LACTOSE	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
CELL SHAPE	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
<u>GRAM REACTION</u>	<u>±</u>	<u>±</u>	<u>±</u>	<u>±</u>	<u>±</u>	<u>±</u>	<u>±</u>	<u>±</u>	<u>±</u>	<u>±</u>	<u>±</u>

Table 3 Biochemical Test for Microbial Identification and Probable Identity of Isolates

PARAM ET ERS	Met h yl red	Voges proseka u er	Cat al ase	Sulp h ide	Indol e	Motil ity	Gluco se	Lact ose	Cell shape	Gram reactio n	Probable Identity
C1	-	+	-	-	-	-	AG	NC	Coc ci	+	<i>Leuconostoc spp.</i> or <i>Lactococcus lactis</i>
C2	-	+	-	-	-	-	A	A	Rod	+	<i>Lactobacillus spp.</i> (homofermentati ve)
C3	-	+	+	+	-	+	A	A	Rod	+	<i>Bacillus subtilis</i> or <i>B. licheniformis</i>
C4	-	+	+	+	-	+	AG	A	Rod	-	<i>Enterobacter spp.</i> or <i>Pantoea spp.</i>
C5	-	+	+	+	-	+	A	a	Rod	+	<i>Bacillus spp.</i>
C 6	-	-	+	+	-	+	NC	NC	Rod	-	<i>Pseudomonas spp.</i> (glucose NC)
C7	-	+	+	+	-	-	NC	A	Rod	-	<i>Acinetobacter spp.</i>
C8	-	+	+	+	-	-	A	NC	Rod	-	<i>Citrobacter spp.</i>
C9	-	-	+	+	-	-	A	NC	Coc ci	+	<i>Lactococcus spp.</i>
C10	-	-	+	+	-	-	A	NC	Rod	+	<i>Bacillus spp.</i> <i>Bacillus cereus</i>
C11	-	+	+	+	-	-	A	a	Rod	+	group
C12	-	+	+	+	-	-	NC	NC	Rod	+	<i>Bacillus spp.</i> <i>Bacillus cereus</i> or <i>Clostridium spp.</i>
C13	-	+	+	+	-	+	NC	NC	Rod	+	<i>Bacillus subtilis</i> group
C14	-	+	+	+	-	+	NC	NC	Rod	+	<i>Bacillus spp.</i>
C15	-	+	+	+	-	+	NC	NC	Rod	+	<i>Bacillus spp.</i>
C16	-	+	+	+	-	+	NC	NC	Rod	+	<i>Bacillus spp.</i>
C17	-	+	+	+	-	-	NC	NC	Rod	+	<i>Bacillus spp.</i>
C18	-	+	+	+	-	-	NC	NC	Rod	+	<i>Bacillus spp.</i>
C19	-	+	+	+	-	-	NC	NC	Rod	+	<i>Bacillus spp.</i>
C20	-	+	+	+	-	-	NC	NC	Rod	+	<i>Bacillus spp.</i>
C21	-	+	+	+	-	-	NC	NC	Rod	+	<i>Bacillus spp.</i>
C22	-	+	+	+	-	-	NC	NC	Rod	+	<i>Bacillus spp.</i>

Table 4 : BLAST Predictions on the NCBI Database

Name of Sample	BLAST prediction
Yogurt 1	<i>Lactobacillus acidophilus</i>
Yogurt 2	<i>Limoilactobacillus reuteri</i>
Yogurt 3	<i>Leuconostoc mesenteroides</i>
Yogurt 4	<i>Lactococcus lactis</i>

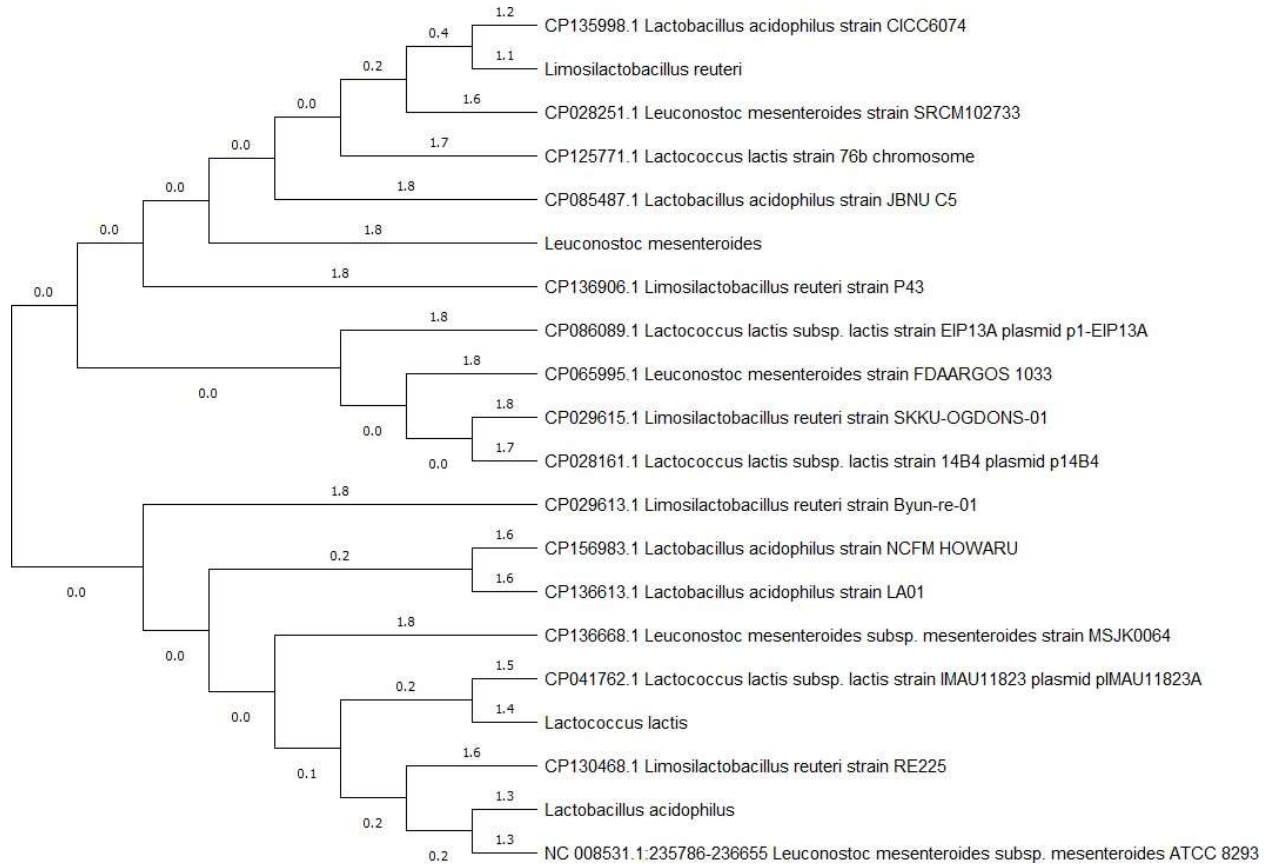
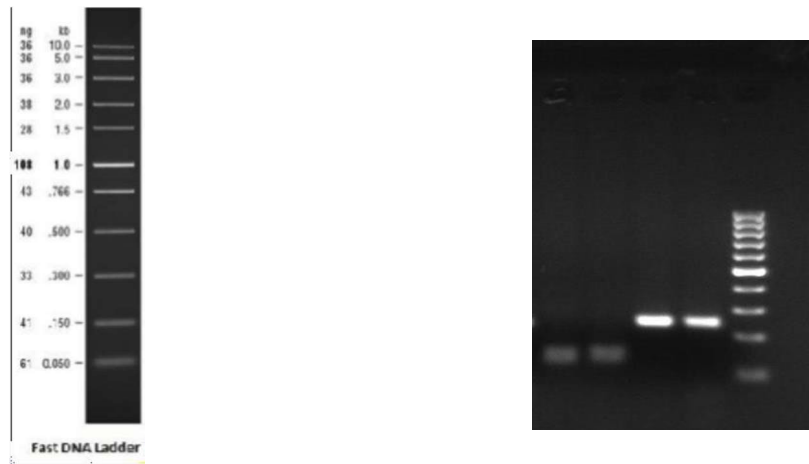


Figure 1: Phylogenetic Tree of Lactic Acid Bacteria Isolates Based on 16S rRNA Gene Sequences

RESULTS:

Figure1: A photographic image of an agarose gel indicting the amplification of the 16S target



Annotation of ladder used in

Figure 2: A photographic image of an agarose gel indicting the amplification of the 16S target

Discussion

The microbiological analysis of the raw coconut milk samples in this study revealed the microbial profile of the milk as having bacterial isolates. A total of 22 isolates were recovered and characterized using Gram staining, cell morphology, and a battery of biochemical tests. These isolates included both Gram-positive and Gram-negative bacteria, with a predominance of rod-shaped, catalase-positive, Voges-Proskauer (VP)-positive organisms. Many of the isolates belong to genera that are well-known for their roles in fermentation, spoilage, or environmental persistence. The initial group of isolates (C1–C11) included species presumably identified as *Leuconostoc sp.*, *Lactobacillus sp.*, *Lactococcus sp.*, *Bacillus sp.*, and a few Gram-negative genera like *Pseudomonas sp.*, *Enterobacter sp.*, *Citrobacter sp.*, and *Acinetobacter sp.* These findings are consistent with previous studies on the natural microbiota of plant-based milk, especially coconut milk. For instance, Ajogun et al. (2023) and Qadi et al. (2023) both reported the presence of lactic acid bacteria (LAB) such as *Lactobacillus spp.* and *Leuconostoc*

spp. in freshly expressed and fermented coconut milk, confirming their natural occurrence and potential for fermentation. Similarly, Gengan et al. (2024) observed a mixed microbial population in Malaysian coconut cultivars, highlighting variability based on breed and handling conditions. Isolates such as C1, identified as *Leuconostoc spp.* or *Lactococcus lactis*, and C2, likely a homofermentative *Lactobacillus* are of interest. These genera are staples in traditional dairy fermentation and have been successfully applied in plant-based substrates to produce desirable acidification and flavor profiles. Their presence in raw coconut milk suggests that the substrate is naturally predisposed to fermentation, especially when handled under hygienic conditions and guided with starter cultures.

However, the detection of Gram-negative rods such as *Pseudomonas spp.* (C6), *Enterobacter spp.* (C4), and *Citrobacter spp.* (C8) indicates possible environmental contamination or poor post-harvest hygiene, which aligns with concerns raised by Sanful (2009) in his early work on promoting coconut milk for yogurt production. These genera, although not necessarily pathogenic in small numbers, are not desirable in fermented food due to their spoilage potential and competition with beneficial fermentative microbes. Their presence reinforces the need for starter culture inoculation and pasteurization during coconut milk yogurt preparation.

The second group of isolates (C12–C22) was more homogenous, consisting entirely of Gram-positive, catalase-positive, VP-positive, rod-shaped, non-sugar fermenting bacteria a classic biochemical signature of *Bacillus sp.* These organisms are spore-forming and can survive harsh environmental conditions, making them common contaminants in raw plant materials. While *Bacillus cereus* (C13) is a known opportunistic pathogen, others like *B. subtilis* and *B. megaterium* have potential biotechnological and probiotic applications, as highlighted in the work of Peters et al. (2023) and Vitheejongjaroen et al. (2024). The latter study, in particular, evaluated the probiotic viability of *Lactocaseibacillus paracasei* in coconut milk, demonstrating that carefully selected strains can dominate the microbial ecosystem and improve safety and shelf-life. The use of MRS agar and serial dilution techniques proved to be effective in isolating and purifying LAB from uncontrolled fermentation coconut milk. The molecular analysis using 16S rRNA gene sequencing provided a reliable means of identifying the LAB isolates at the species level. Our results demonstrated that the LAB isolates shared high sequence similarity with reference strains, indicating that they are closely related to known LAB species. The

isolated LAB species have potential applications in the production of non-dairy yoghurt products. *Lactobacillus acidophilus* and *Limosilactobacillus reuteri*, for example, are known to possess probiotic properties and can confer health benefits to consumers. *Leuconostoc mesenteroides* is a versatile species that can be used as a starter culture in various food fermentations. *Lactococcus lactis* is a widely used starter culture in dairy and non-dairy fermentations.

Conclusion

This study successfully isolated and identified four species of lactic acid bacteria (LAB) from fermented coconut yoghurt, namely *Lactobacillus acidophilus*, *Limosilactobacillus reuteri*, *Leuconostoc mesenteroides*, and *Lactococcus lactis*. The molecular analysis using 16S rRNA gene sequencing confirmed the identity of these isolates and demonstrated their close relationship to known LAB species. The use of isolated LAB species as starter cultures can help to improve the quality and consistency of the final product.

Recommendations

Based on the findings of this study, the following recommendations are proposed for researchers, food producers, public health authorities, and other stakeholders interested in plant-based probiotic products and food safety: Adoption of Coconut Milk as a functional Yoghurt base, use of defined starter cultures for safety and consistency, improved sanitary handling and pasteurization of raw Coconut Milk.

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