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Phenotypic Characterisation and Molecular Identification of Potentially Probiotic *Lactobacillus* sp. Isolated from Fermented Rice

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Abstract: Fermented rice is known as a healthy food due to the presence of lactic acid bacteria. The study was carried out to identify and characterise the lactic acid bacteria (LAB) from white and red fermented rice Bg (Bathalagoda) varieties. Fermentation was carried out naturally by soaking red, white, raw, and cooked rice in sterile distilled water (1:3) overnight at 27 °C in an earthen pot. Potentially probiotic bacterial were isolated and the species of the isolated lactic acid bacteria were confirmed based on 16S rDNA gene sequencing and were studied for phenotypic characteristics, including morphological, physiological (growth temperature, salt tolerance, milk coagulation), and biochemical (carbohydrate fermentation pattern) characteristics, using API 50CH kits. Distinct clusters of cocci (48), diplococci (30), and rod-shaped bacteria (30) were observed in fermented rice. Five species of lactic acid bacteria were identified, including *Latilactobacillus curvatus* GRLb1, 2, 10, and 11 (the predominant Bacillus species); *Latilactobacillus graminis* GRLb 8; *Limosilactobacillus fermentum* GRLb17; *Weissella confuse* GRLb4; *and Pediococcus pentosaceus* GRLc1. The base pair length of amplified DNA for the isolates was 1500 Bp. Most of the isolates were able to grow at temperatures ranging from 10 °C to 45 °C, tolerate up to 6.5% salt, and coagulate milk with homofermentative characteristics. The beneficial physiological and biochemical properties of isolated *Lactobacillus* species from fermented rice revealed their potential applications in the food industry. The similar species of bacteria that were isolated from different sources show their probiotic characteristics. Further studies are recommended to confirm their probiotic properties and health benefits.

Keywords: fermentation; 16S rDNA; lactic acid bacteria; phenotypical; genotypical

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1. Introduction

Fermentation is a simple and economical way of preserving food, improving the nutritional value, sensory properties, and functional qualities of food, and it can also be applied in the production of functional foods such as probiotic foods [1]. Fermented foods are produced worldwide using various manufacturing techniques, raw materials, and microorganisms [2]. Fermented foods offer beneficial health effects due to the antimicrobial

effects of the lactic acid bacteria present in them. Due to their interesting beneficial properties, LAB are widely used as starter cultures, probiotics, and microbial cell factories for the production of bioactive components, including lactic acid, which shows an antimicrobial effect and interferes with the growth of pathogenic microorganisms [3]. The production of functional foods prepared using non-dairy-based materials can satisfy the dietary requirements of consumers who have allergies to dairy-based products [4]. Microorganisms possess probiotic activity that promotes a positive health image during fermentation [5]. Fermented foods can be consumed by vulnerable groups such as children, expectant and breastfeeding mothers, the elderly, sick people, and recovering people for good health [6].

In the past, farmers and labourers from Asian countries kept leftover rice for fermentation overnight and consumed for breakfast, which has been known to offer several health benefits. This food provides sustained energy with all-natural nutritional supplements that arise from the fermenting bacteria inside of it. Consumption of fermented rice is believed to have a soothing effect on the intestines because the rice is fermented and digested by bacteria. Bacteria add fibre to stool and also produce vitamin B complex (B12), vitamin D, and vitamin K. Fermented rice is locally called 'pazhaya sadham' in India and is prepared by soaking cooked parboiled rice in water overnight. Fermentation enhances the bioavailability of essential nutrients, especially minerals, through the enzymatic reduction of phytate [7]. Fermented rice is one of the neglected foods in countries where rice is the staple food. The consumption of fermented rice as a contributor to a healthy lifestyle is either ignored or ridiculed as a poor man's lifestyle food. Many strains of probiotic lactic acid bacteria have been isolated from different traditional fermented foods; insufficient research data are available on studies that are connected to the identified lactic acid bacteria from fermented rice. Exploring the natural (wild) bacteria responsible for the fermentation of rice is essential for confirming the health benefits of fermented food products. *Pedicoccus pentosaceus* and *Lactobacillus plantarum* are potentially probiotic bacteria that are isolated from fermented rice gruel prepared from the 'Ponni' variety of rice (*Oryza sativa*) [8]. Parboiled rice and black grams are generally used in Sri Lanka for cooking and for producing rice-based fermented products [9]. Fermented white and red Batalagoda (Bg) rice varieties are used for the preparation of fermented foods such as hoppers, 'Idli', and 'Dosai'. The present study was aimed at distinguishing the responsible microorganisms for the fermentation of red and white rice Bg varieties.

Lactic acid bacteria (LAB) constitute a diverse group of industrially important and safe bacteria; they have interesting beneficial properties that are primarily being used as starter cultures, probiotics, and microbial cell factories for the production of highly valuable bioactive components [10]. Exploring the LAB present in unknown niches may identify unique species or strains with pertinent technological and probiotic properties. Autochthonous rather than allochthonous starter cultures are chosen in the contemporary industry of fermented food products due to the better adaptation and performance of autochthonous strains to the matrix that they originate from. The wide distribution and important nutritive value of cereals have led to a focused attention on cereal as a raw material for the development of new fermented functional foods. Most probiotic foods available worldwide are milk-based, and few attempts have been made to develop probiotics using cereals as the substrate. LAB isolated from fermented rice has the potential to be used as a starter culture in cereal-based fermented food products or probiotic foods.

The identification of lactic acid bacteria based on phenotypic characterisation is still being used as a starting point prior to the molecular characterisation of the key characteristics in bacterial taxonomy [11]. Seven predominant Lactobacillus species and one coccus species were isolated from fermented white and red raw and cooked rice (Bg varieties), and they were identified at the genus level [12]. These novel Lactobacillus species should be confirmed by molecular studies. Therefore, the current study was carried out to recognise the species of lactic acid bacteria isolated from fermented rice based on 16S rDNA gene sequencing and phenotypic characterisation.

2. Materials and Methods

2.1. Study Location

The isolation and characterisation procedures of *Lactobacillus* sp. from fermented rice were carried out at the Department of Oral Medicine and Periodontology, Faculty of Dental Sciences, University of Peradeniya, Sri Lanka; molecular identification via 16S rDNA sequencing was carried out at the Department of Food Technology, University Putra, Malaysia (UPM).

2.2. Materials

Fermented red and white raw rice and cooked rice samples (Batalagoda (Bg) rice varieties, red and white) were used as isolation sources, which were purchased from CIC Agri., Kandy, Sri Lanka. MRS (deMan Rogosa and Sharpe, Oxide, UK) agar, MRS agar with (0.2%) sorbitol, and MRS agar with (0.25%) L-cysteine were used in order to improve the specificity of the medium for the isolation of diverse species of lactic acid bacteria. The pH of the media was measured using a pH meter (HM-7E, Japan). The pH of the MRS agar, MRS sorbitol (0.2%), and MRS with L-cysteine (0.25%) were 6.2, 6.8, and 5.8, respectively. Direct samples and dilutions (0.1 mL aliquots) were inoculated onto the plates using the spread plate method. The inoculated plates were incubated at 37 °C for 72 h under an anaerobic condition using an anaerobic sachet (Anaerogen, Oxoid) with replications. The culture was subsequently streaked 4–5 times to obtain a pure culture.

The eight predominant bacterial isolates that survived, including seven rod-shaped (Isolation codes Lb-1, Lb-2, Lb-4, Lb-8, Lb-10, Lb-11, Lb-17, and Lc-1) and one spherical-shaped bacteria, were studied for phenotypic characterisation and molecular identification.

2.3. Preparation of Rice Samples

The red raw and white rice (*Oryza sativa*) were cleaned and washed twice and separately cooked in water (rice:water; 1:3) for 30 min to obtain a soft consistency. The fermentation was carried out naturally by soaking raw or cooked rice (50 g) in sterile distilled water (rice:water; 1:3) overnight in earthen pots at 27 °C in an outside environment. Cooked and raw rice samples of each variety of rice were kept in separate pots; samples were collected from each pot.

2.4. Phenotypic Characterisation

Isolates were identified by their phenotypic characterisation. The phenotypic characterisation was carried out by studying the morphological, physiological, and biochemical characteristics based on Barrow and Felthman [13], and these bacteria were identified at the species level using their phenotypic features and carbohydrate fermentation patterns as observed in an API 50 CHL system.

2.4.1. Morphological Characteristics of the Isolates

The methods for Gram staining, catalase activity, motility, and spore formation were followed according to Barrow and Felthman [13]. The morphological structure of isolates was observed with the light microscope using an oil immersion lens (10 * 100) following Gram staining.

2.4.2. Physiological and Biochemical Characterisation

Culture Preparation

A total of 2 to 3 colonies of the 8 isolates were inoculated in MRS broth containing (0.25%) L-cysteine (10 mL) and incubated at 37 °C for 18 h to obtain a fresh culture. The experiments to study the growth at different temperatures and salt concentrations, milk coagulation, and gas production assays were carried out in replicates.

Growth at Different Temperatures

The growth at different temperatures was evaluated with some modifications, as described in Hatice [14]. MRS broth containing (0.25%) L-cysteine containing Bromocresol purple (0.04 g/L) indicator was prepared as the test medium, and 5 mL of the test medium was transferred into clear glass test tubes for observation. Fresh cultures (50 µL, 18 h, McFarland standard 0.5) were transferred into the test medium and incubated for 7 days at 10 °C, 37 °C, 40 °C, 45 °C, and 55 °C in an incubator (Thermo scientific, Herathem, Germany). The temperature ranges were selected based on the optimum growth temperatures of the *lactobacillus* species. During incubation, microbial growth was confirmed by the colour change of the cultures from purple to yellow. The test medium inoculated with the isolated culture and incubated at 37 °C was considered as a positive control, and the without inoculated cultures that were incubated at each tested temperature were used as negative controls. The McFarland standard was used to compare the growth.

Growth at Different NaCl Concentrations

The salt tolerance of bacteria was used to characterise the lactic acid bacteria for identification (Barrow and Felthman, 1995 [13]). Isolates were tested for salt tolerance in different NaCl concentrations (Sigma-Aldrich, Burlington, MA, USA) based on the method described by Hatice [14] with some modifications. The NaCl concentrations of 2, 4, 6.5, and 10% were selected. Test media containing bromocresol purple (5 mL) indicator were prepared at appropriate concentrations (0.04 g/L) and transferred into clear glass tubes, which were inoculated with 1% (*v*/*v*) fresh culture (McFarland standard 0.5) and then incubated at 37 °C for 7 days. The cell growth was confirmed by the colour change from purple to yellow. MRS broth containing (0.25%) L-cysteine, isolated cultures, and 0% NaCl was used as a positive control. The negative control (broth without inoculated culture) is included to confirm the contamination.

Gas Production with Glucose

Carbon dioxide production from glucose was observed in order to determine the homofermentative and heterofermentative characteristics of the eight isolates. The test medium was prepared by adding MRS broth with (0.25%) L-cysteine, without citrate, and broth inoculated with 1% (*v*/*v*) fresh cultures (18 h). Durham tubes were kept in an inverted position into the test medium, which were incubated at 37 °C for 7 days. The carbon dioxide production from glucose was confirmed by gas accumulation in the Durham tubes. The test medium prepared without culture was used as a control [14].

Milk Coagulation Assay

The milk coagulation assay in skim milk was performed as previously described by Marokki et al. [15] with some modifications. Isolates were introduced into MRS L-cysteine broth and incubated at 37 °C for 16–18 h for activation. The culture (1%, *v*/*v*) was transferred into culture tubes containing sterile skim milk (12.5%), and the coagulation of the milk was determined after 16 h of incubation at 37 °C. The probiotic mixed starter culture (ABT-3, Hansion Denmark, *Lactobacillus acidophilus* LA5, *Streptococcus thermophilus* TH4, and *Bifidobacterium bifidum* Bb 12) inoculated with skim milk and skim milk without the culture were used as positive and negative controls, respectively.

2.5. Carbohydrate Fermentation Pattern of Isolates on the API 50 CHL System

The isolates were identified at the species level using API 50 CH kits based on the carbohydrate fermentation profile of CHL media (Biomérieux, Marcy-l'Étoile, France) by following the manufacturer's instructions.

2.5.1. Preparation of the Culture

Several identical colonies were picked up from MRS culture plates, incubated at 37 °C for 2 days, and introduced into the culture tubes containing sterile distilled water (2 mL) for

the preparation of a heavy culture suspension. The suspension was added into the culture tubes containing sterile distilled water (5 mL) to prepare a suspension with a turbidity that was equivalent to McFarland standard 2, and the number of drops required was recorded (n). The API 50 CHL medium (10 mL) was inoculated by transferring the number of drops of the prepared suspension (2n) twice into the API 50 CHL Medium ampule. This inoculated medium was mixed well and used immediately.

2.5.2. Preparation of the Incubation Box

The references of the isolates were marked on the elongated flap of the tray, and sterile distilled water (10 mL) was added into the honeycombed wells of the tray to create a humid atmosphere.

2.5.3. Preparation of the Strips

Each API strip was composed of 5 smaller strips, each containing 10 numbered tubes. The strips were cut into 5 smaller strips (0–9, 10–19, 20–29, 30–39, and 40–49) and placed in the proper order in the incubation tray.

2.5.4. Inoculation of the Strips

The inoculated API 50 CHL medium was distributed using a sterile pipette among 50 tubes without the formation of bubbles, and all tubes were covered with mineral oil to maintain anaerobic conditions. The inoculated stripes were incubated at 37 °C for 48 h.

2.5.5. Reading and Interpretation

The strip was read for positive (+) and negative reactions (−) after 48 h of incubation, and the results were recorded on the result sheet. A positive test corresponds to acidification, which was shown by the bromocresol purple indicator contained in the API medium changing from purple to yellow, except in the esculin test (tube no. 25), where a change in colour from purple to black was considered positive. The biochemical profile obtained for the strain can be identified using the apiweb^{TM} identification software with database (V5.1). The API profiles were analysed (Figure A1) using apiweb^{TM} identification software V5.1 (Biomérieux, Marcy-l'Étoile, France). In cases where two or more significant taxa were indicated, reference was made to standard texts for identification by considering their physiological characteristics.

2.6. Identification of Lactobacillus sp. by 16S rDNA Sequencing

2.6.1. Extraction of Genomic DNA

The overnight MRS broth culture (maximum 2×10^9 cells) was harvested in a microcentrifuge tube by centrifuging for 10 min at 5000× *g*, and the supernatant was discarded. The bacterial pellets were used for the extraction of DNA. DNA was extracted using a DNA extraction kit (QIAGene, Hilden, Germany) by following the manufacturer's protocol with some modifications. Extracted DNA (3 µL of DNA) was mixed with 2 µL of 6x loading dye, visualised by agarose gel (1.5%) electrophoresis under UV light, and documented using the Gel Doc system. The extracted DNA were then taken to the PCR step.

2.6.2. Polymerase Chain Reaction for the Amplification of the 16S rDNA Region

The most common primer pair devised by Weisburg et al. [16] was forward primer 27F and reverse primer 1492R, which are currently referred to as universal primers. The forward primer is complementary to the 5′ end of 16S rDNA, and the reverse primer is complementary to the 3′ end of the 16S rDNA region. Amplification of the 16S rDNA region was carried out using universal primers, including forward primer 27F (5′-AGAGTTTGATCCTGG CTCAG-3′) and reverse primer 1492R (5′-GTTACCTTGTTACGACTT-3′) [17].

Amplification reactions were performed in a total reaction volume of 50 µL containing 2X Taq Master Mix (25 µL) (New England Biolabs Inc., Ipswich, UK), 0.25 mM of forward primer (1 µL), 0.25 mM (1 µL) of reverse primer (1 L) (First BASE, Serdang, Malaysia),

deionised water (22 µL), and genomic DNA. The 1 µL of genomic DNA was mixed with 49 µL of PCR mixture, and the final reaction mixture was taken for the PCR steps. A negative DNA control was performed by adding 1 µL of nuclease-free water to the PCR mixture instead of genomic DNA. The preliminary experiments were carried out to confirm the amplification of the DNA. Gradient temperature PCR (Eppendorf, Homburg, Germany) was carried out with the initial heating of 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 55 s, extension at 72 °C for 1 min, and termination at 10 min of final incubation at 72 °C.

2.6.3. Separation of Amplified PCR Products

Preparation of agarose gel: Agarose gel (Vivantis Biotechnology, Kuala Lumpur, Malaysia) was prepared by dissolving 0.8 g of agarose in 100 mL of boiling TAE buffer (1X), cooling the solution to nearly 45 °C, and adding 1.5% RedSafe Nucleic Acid Staining Solution (concentration 20,000X, Intron Biotechnology, Seongnam-si, Korea). The prepared agarose gel was poured into the gel casting stand, and the combs were placed. The combs were taken out after the formation of rigid gel within the well. The PCR products (5 µL) and 2 µL of the loading dye (QIAGene, Hilden, Germany) were mixed and loaded into wells. A DNA size marker (2 µL) (10 kb + 100 bp, Fermentas, Burlington, ON, Canada) was loaded into the first well to observe the right amplified region.

Electrophoresis of the products: Extracted DNA and PCR products were electrophoresed at 180 V.80 mA for one hour in the gel electrophoresis system (Major Science, Taoyuan, Taiwan). Amplified band patterns were visualised in a UV transilluminator (Major Science, Taiwan) and documented using the Gel Doc system (UVIdoc System, model GAS9000/9010).

2.6.4. Sequencing and Phylogenetic Tree Development

The amplified 16S rDNA PCR products were sequenced by First BASE laboratories, Malaysia (First BASE, Kuala Lumpur, Malaysia) using 27F and 1492R primers corresponding to the positions of forward primer 27F (5′-AGAGTTTGATCCTGG CTCAG-3′) and reverse primer 1492R (5′-GTTACCTTGTTACGACTT-3′). The sequences of reverse primer 1492R were converted into their reverse order in reverse complement. The sequences of the isolates were edited using BioEdit 7.2 software. The sequences with the highest QV were selected, and both reverse and forward sequences were combined. Sequence homologies of the isolates were examined by comparing the sequences obtained with 16S rDNA and sequences deposited in the nucleotide databases of the GenBank (NCBI) using the basic local alignment search tool (BLAST) program, and gene accession numbers were obtained.

The representative sequences of the Lb-1, Lb-2, Lb-4, Lb-8, Lb-10, Lb-11, Lb-17, and LC-1 isolates were then aligned using ClustalW of the MEGA 11 package, and the phylogenetic tree was built using the neighbour-joining method [18]. The stability of the tree was assessed by the bootstrap method using 1000 replications.

3. Results

3.1. Morphological Characteristics of Isolates

After Gram staining, the cell morphology of all isolates was observed using the oil immersion objective (10 × 100) of a light microscope. Among the studied colonies (Figure A1), the majority of the isolated microorganisms from fermented rice appear to be clusters of cocci and rod-shaped bacilli.

Three different morphotypes of the isolates, including cluster coccus (48), diplococcus (30), and rod (30) (Figure 1, Tables S2–S4), were observed with Gram-positive and catalase-negative reactions in all three media. Coccus-shaped species was predominantly observed in MRS media arising from red and white raw rice while rod-shaped bacteria was predominant in the MRS L-cysteine broth (0.25%). The number of studied colonies shows that the majority of the isolated microorganisms from fermented rice appear to be clusters of cocci and rod-shaped bacilli. A subsequent (3–4) purification process was attempted for survived isolates, and then rod-shaped bacteria and the predominant cluster-like coccus

were tested for motility and endospores. Seven rods and cluster-like coccus were identified as lactic acid bacteria as they were Gram-positive, catalase-negative, non-motile, and did not form endospores; they were preserved in MRS broth containing 20% (*v*/*v*) glycerol and stored at −80 °C as frozen cultures for long-term usage.

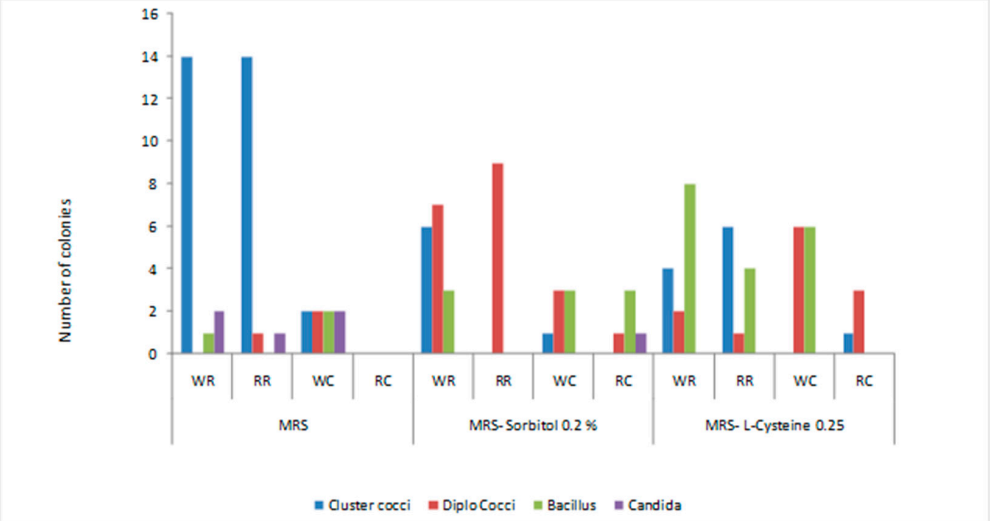


Figure 1. Gram-positive, catalase-negative bacteria, and *Candida* sp. in the MRS and MRS-modified media from fermented rice. WR—white raw; WC—white cooked; RR—red raw; and RC—red cooked fermented rice.

Four morphologically distinguished types of lactobacilli were observed, including short, medium, long, and very long regular rods that appeared as single, pair, or V-shaped short chains (Table 1), and coccus appeared as clusters of similar morphological appearance.

Table 1. Morphological characteristics of isolated lactic acid bacteria from fermented rice.

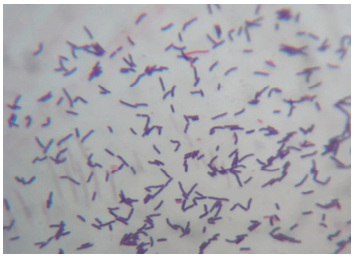
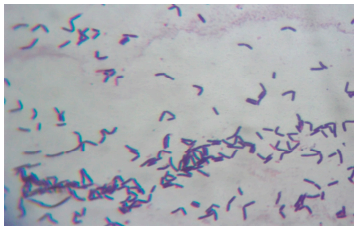
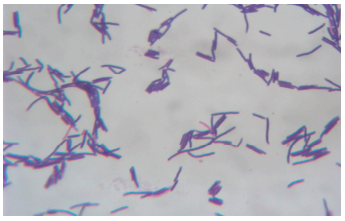
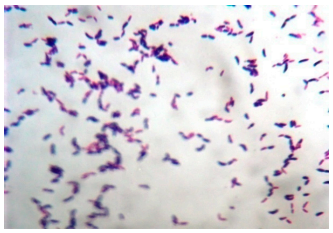
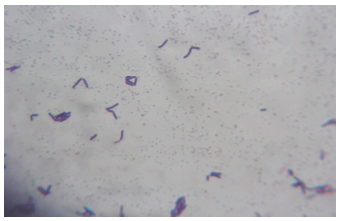
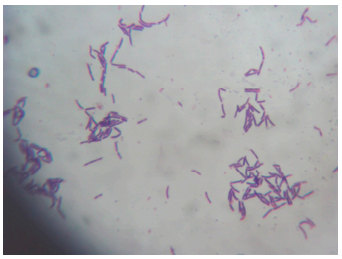
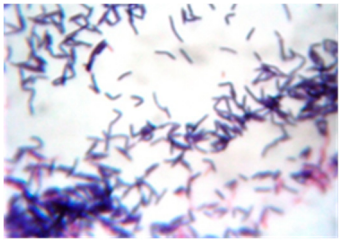
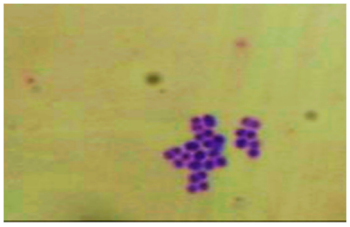
Isolates	Lactic Acid Bacteria	Morphological Description	Appearance under Light Microscope (×1000 Magnification)
Lb1	*Latilactobacillus curvatus*	Medium size, rod (regular)-shaped bacteria, arranged as single/pair or as a group in a 'V' arrangement	
Lb2	*Latilactobacillus curvatus*	Medium size, rod (regular)-shaped bacteria, arranged as single/pair or as a group in a 'V' arrangement	

Table 1. *Cont.*

Isolates	Lactic Acid Bacteria	Morphological Description	Appearance under Light Microscope (×1000 Magnification)
Lb4	*Weissella confusa*	Very long size, rod (regular)-shaped bacteria, arranged as single/pair or as a group in a 'V' arrangement	
Lb8	*Latilactobacillus graminis*	Small size, rod (regular)-shaped bacteria, arranged as single/pair or as a group in a 'V' arrangement	
Lb10	*Latilactobacillus curvatus*	Medium size, rod (regular)-shaped bacteria, arranged as single/pair or as a group in a 'V' arrangement	
Lb11	*Latilactobacillus curvatus*	Medium size, rod (regular)-shaped bacteria, arranged as single/pair or as a group in a 'V' arrangement	
Lb17	*Limosilactobacillus fermentum*	Long size, rod (regular)-shaped bacteria, arranged as single/pair or as a group in a 'V' arrangement	
Lc1	*Pediococcus pentosaceus*	Coccus-shaped bacteria, arranged as single, tetrad, or group/cluster	

3.2. Physiological and Biochemical Characterisation

Lactobacillus are mostly used as starter cultures for industrial purposes. Seven rod-shaped bacteria were selected for physiological characterisation (Table 2).

Table 2. Physiological and biochemical characterisation of the isolates.

Physiological and Biochemical Characteristics	Lb-1	Lb-2	Lb-4	Lb-8	Lb-10	Lb-11	Lb-17	Positive	Negative
Gram test	+	+	+	+	+	+	+	NA	NA
Catalase test	−	−	−	−	−	−	−	NA	NA
Motility	−	−	−	−	−	−	−	NA	NA
Spore formation	−	−	−	−	−	−	−	NA	NA
Gas from glucose	−	−	−	+	−	−	−	ND	−
Growth at 10 °C	++	++	++	+	++	++	++	NA	−
Growth at 37 °C	++	++	++	++	++	++	++	++	−
Growth at 40 °C	++	++	++	++	++	++	++	NA	−
Growth at 45 °C	+	+	+	+	+	+	+	NA	−
Growth at 55 °C	−	−	−	−	−	−	−	NA	−
0% Nacl	++	++	++	++	++	++	++	++	
2% NaCl	++	++	++	++	++	++	++	NA	−
4% NaCl	++	++	++	++	++	++	++	NA	−
6.5% NaCl	++	++	++	++	++	++	++	NA	−
10% NaCl	−	−	−	−	−	−	−	NA	−
Milk coagulation	+	+	+	+	+	+	+	+	−

NA—Not applicable; ND—not determined; ++: heavy growth (MacFarland standard >1.0); +: growth (MacFarland standard −1.0); − no growth.

3.2.1. Growth at Different Temperatures

Different temperatures (10 °C, 37 °C, 40 °C, 45 °C, and 55 °C) were tested as the optimum incubation temperature for yoghurt production. The positive growth and survival of isolates were observed for all isolates at temperatures of 10 °C, 37 °C, 40 °C, and 45 °C, with the colour changing from purple to yellow. (Table 2). A higher growth turbidity was visually observed in the isolates Lb-1, 2, 4, 10, 11, and 17 (*Latilactobacillus curvatus* strain GRLb-1, 2, 4, 11, and 17) at a temperature range of 10–40 °C compared with the turbidity at 45 °C. The growth temperature of 10 °C is a positive attribute of the probiotic *Lactobacillus* sp., and there may be the possibility of maintaining the viable count of the probiotic at the therapeutic minimum count (10^7 cfu/mL or g of carrier substrate) during refrigerated storage. The isolate Lb-8 (*Latilactobacillus graminis* strain GRLb8) grew densely at 37 °C and 40 °C. All isolates preferred mesophilic temperatures to thermophilic temperatures for growth. The positive control at 37 °C showed heavy growth with a yellow colour, while the negative control without culture appeared as a purple colour even after 7 days of incubation. Sustaining the optimum growth temperature is important for producing microbial products in bioreactors; furthermore, the growth temperature is an important physiological characteristic for the identification of novel bacteria from new sources.

Generally, different species of the genus Lactobacillus can tolerate temperatures ranging from 15 °C to 45 °C. *Lactobacillus acidophilus*, *Lactobacillus delbruki*, and *Lactobacillus salivarius* can grow at 45 °C and are unable to grow at 15 °C, while *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus berevis* can grow at 15 °C and cannot grow at 45 °C [13].

3.2.2. Growth at Different NaCl Concentrations

It is observed that all cultures showed positive heavy growth at 0, 2, 4, and 6.5% NaCl concentrations with the yellow colour change of the media. However, no growth was observed for all cultures at the 10% NaCl concentration (Table 2). The negative control (devoid of culture) did not show any growth, and the colour of the medium remained purple. In a previous study, Lactobacillus species isolated from Algerian goat milk showed a 2% and 4% salt tolerance (Marroki [15]). The tolerance to a 6.5% NaCl concentration is beneficial as a starter culture in the fermentation process, and it should survive in a fermentation environment with a high salt concentration [19].

3.2.3. Gas Production with Glucose

In order to determine the homofermentative or heterofermentative nature of the cultures, all isolates were studied for gas production from glucose. The isolate Lb-8 (*Latilactobacillus graminis* GRLb8) produced gas during the fermentation of glucose, showing a heterofermentative nature, while others did not release gas from glucose and showed a homofermentative nature (Table 2). The genus Lactobacillus can be either heterofermentative or homofermentative (Barrow and Felthman [13]).

Homofermentative organisms ferment glucose into two moles of lactic acid, generating a net of 2 ATP per mole of glucose metabolised. Lactic acid is the major product of this fermentation. Heterofermentative lactic acid bacteria ferment one mole of glucose into one mole of lactic acid, one mole of ethanol, and one mole of CO2. One mole of ATP is generated per mole of glucose, resulting in less growth per mole of glucose metabolised. Because of their low energy yields, lactic acid bacteria often grow more slowly [13].

3.2.4. Milk Coagulation and Curd Formation

Coagulation and curd formation in skim milk were shown by all isolates. LAB generally possesses the ability to coagulate milk due to the production of lactic acid, leading to a decrease in the pH and the presence of a complete proteolytic system consisting of a cell envelope-associated proteinase, which allows for the efficient degradation and utilization of casein [20]. The ability of bacteria to coagulate autoclaved milk within 16 h at 42 °C using 1% freshly coagulated inoculum in cheese production is defined as the fast milk-coagulating (Fmc+) strain [21].

The isolates coagulated the milk within 16 h at 37 °C and can be considered fast milk-coagulating (Fmc+) strains. The coagulation of milk by the isolates revealed their potential as starters or adjunct cultures in the production of fermented dairy food products.

3.2.5. Carbohydrate Fermentation Pattern of *Lactobacillus sp.*

Seven rods and one coccus-shaped bacteria were identified as *Lactobacillus* sp. as they were Gram-positive, catalase-negative, non-motile, and without endospore formations. As per the carbohydrate fermentation pattern of the isolated Lactobacillus species using classical and biochemical tests (using API 50 CHl BioMeriex, Saint-Vulbas, France), seven *Lactobacillus* species and one cluster-like coccus were identified to the species level (Table 3). There were five different *Lactobacillus* sp. and one *Pedicoccus* sp. identified among the tested organisms. Isolates Lb-1, Lb-2, and Lb-11 were identified as *Lactobacillus curvatus* ssp. curvatus with 99% identity, and Lc-1 was identified as *Pediococcus pentosaceus* with 99% identity.

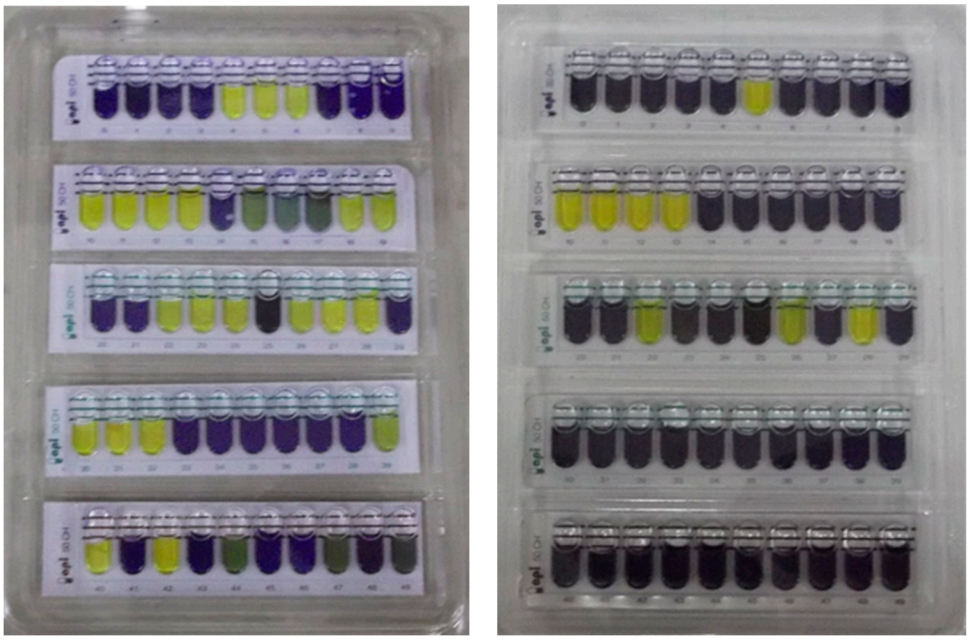
Table 3. Identification of lactic acid bacteria using API 50 kits.

Isolate Code	Species of *Lactobacillus*	* Identity (%)
Lb-1	*Lactobacillus curvatus ssp.curvatus*	98.9
Lb-2	*Lactobacillus curvatus ssp.curvatus*	99.3
Lb-4	*Lactobacillus helveticus*	86.3
Lb-8	*Lactobacillus delbrueckii ssp. delbrueckii*	95.5
Lb-10	*Lactobacillus pentosus*	63.3
Lb-11	*Lactobacillus curvatus ssp.curvatus*	99.3
Lb-17	*Lactobacillus plantrum*	91.3
Lc-1	*Pediococcus pentosaceus*	99.9

* Identity percentage of bacteria using the apiweb™ system.

The carbohydrate fermentation pattern of the isolates showed that all isolates were not able to ferment most of the sugars and were able to only ferment some of the sugars (12–45%) tested using the API 50 kits (Figure 2 and Table S1). All isolates were able to ferment maltose, D-glucose, D-fructose, and D-mannose. The isolate Lb-8 did not ferment N-acetylglucosamine and D-galactose, but all other isolates showed a positive reaction

to them. The carbohydrate fermentation by isolates Lb-1, Lb-2, and Lb-11 showed a similar pattern.



(**a**) (**b**)

Figure 2. Carbohydrate fermentation patterns of Lb-10 (**a**) and Lb-1 (**b**) from fermented rice tested using the API 50 CHL system. The sugar fermentation was identified by the colour change of the bromocresol purple indicator in the API medium to yellow, except in the esculin test (tube no. 25), where a change in colour from purple to black was considered positive. The medium red indicated a negative result for sugar fermentation.

The phenotypic characteristics of microorganisms are useful tools for presumptive classification. The identification of species, especially within the genus Lactobacillus, based on information on the biochemical and metabolic traits of LAB is not confirmative due to the increasing number of lactic acid bacteria species that vary on a small number of biochemical traits [22]. The fermentation ability of some carbohydrates is plasmid-encoded. The loss and acquisition of plasmids lead to metabolite inconsistencies and affect the fermentation pattern [23]. The combination of commercially available API systems with conventional phenotypic methods and genotypic techniques ensures the identification of species with known phenotypic properties.

3.3. Molecular Identification and Genotypic Characteristics of Lactobacillus sp.

3.3.1. Genomic DNA Isolation

Extraction of genomic DNA from isolates was carried out according to the manufacturer's instructions and used for the amplification of the 16S rDNA region by the PCR protocol. Figure 3 shows the extracted DNA from the isolated Lactobacillus species, which confirms the presence of the DNA after the extraction procedure.

3.3.2. Amplification of 16S rDNA Region

After the confirmation of DNA extraction, the extracted DNA was taken up for PCR analysis for the amplification of the 16S rDNA region, and the amplification of the PCR products was visualised using agarose gel electrophoresis under UV light. The amplification of the PCR product bands was observed in the gel documentation system, and the length of the amplification products was nearly 1500 bp (Figure 4).

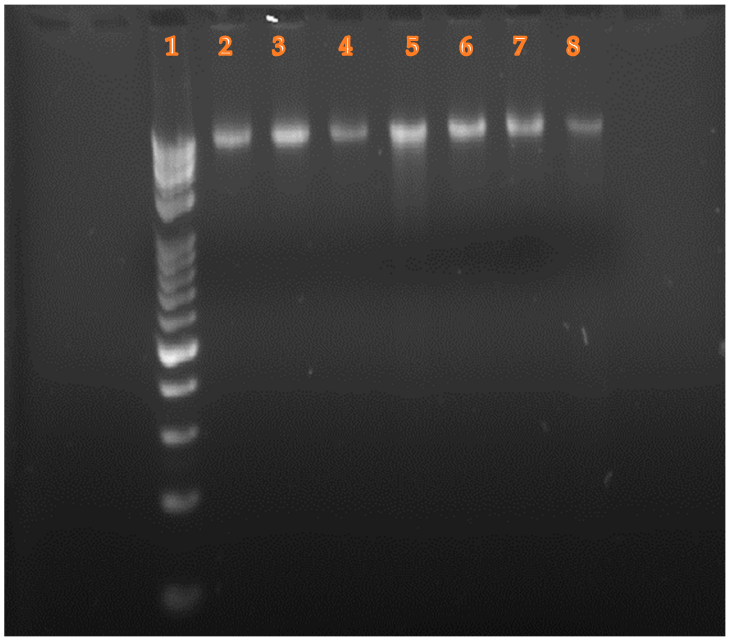


Figure 3. Visualization of extracted DNA by gel electrophoresis. 1. 10 kb + 100 bp DNA ladder; 2. Lb-1; 3. Lb-2; 4. Lb-4; 5. Lb-8; 6. Lb-10; 7. Lb-11; 8. Lb-17. The band pattern in the gel confirmed the presence of extracted DNA.

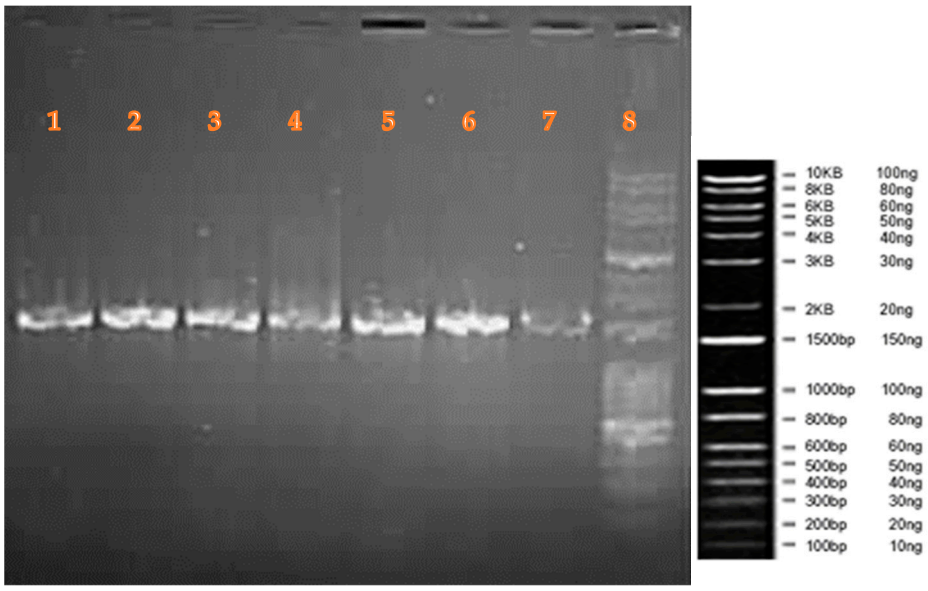


Figure 4. Amplified Products of the 16S rDNA region of the isolates 1. Lb-1; 2. Lb-2; 3. Lb-4; 4. Lb-8; 5. Lb-10; 6. Lb-11; 7. Lb-17; 8. 10 kb + 100 bp DNA ladder.

The base pair length of amplified DNA for all isolates was at a similar level, which stayed at the level of 1500 bp of the DNA ladder.

The length of amplification of the 16S rDNA region depends on the nucleotide sequence. Generally, the length of 16S rDNA fragments obtained from different lactobacilli species was approximately 1500 bp [24]. The PCR of the 16S rRNA genes of *Lactobacillus salivarus* from the blood culture and gall bladder pus showed bands at 1515 bp [25], and the length of the amplified product for *Lactobacillus helvelictus* and Lactobacillus acidophilus isolated from breast milk varied from 1500 to 2000 bp [14].

3.3.3. Identification of LAB Based on Phylogenetic Analyses of 16S rDNA Sequences

The seven rods and one coccus presumptively identified as LAB were subjected to 16S rDNA sequence analyses. The microorganisms were deposited in the Gene Bank (NCBI), and gene accession numbers were obtained (Table 4).

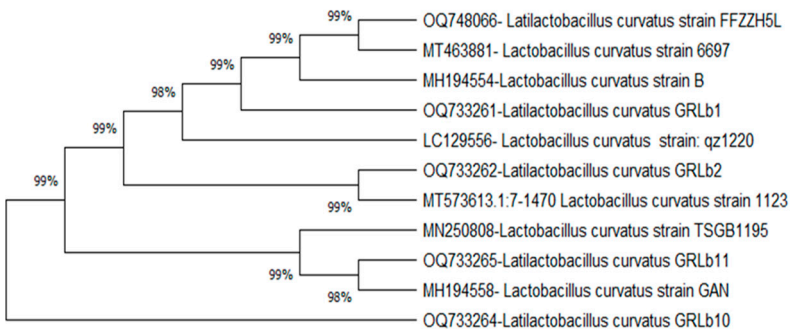
Table 4. Identification of isolated LAB using 16S rDNA sequences.

Sequence ID	Microorganism	Accession Number	Sequence Length
Lb1	*Latilactobacillus curvatus* GRLb1	OQ733261	1475 bp
Lb2	*Latilactobacillus curvatus* GRLb2	OQ733262	1465 bp
Lb4	*Weissella confusa* strain GRLb4	OQ733263	1486 bp
Lb8	*Latilactobacillus graminis* GRLb8	OQ861079	1097 bp
Lb10	*Latilactobacillus curvatus* GRLb10	OQ733264	1469 bp
Lb11	*Latilactobacillus curvatus* GRLb11	OQ733265	1456 bp
Lb17	*Limosilactobacillus fermentum* GRLb17	OQ861078	819 bp
Lc1	*Pediococcus pentosaceus* GRLc1	OQ733260	1480 bp

Based on the closely related strain from BLAST, the isolated LAB were identified down to the species level (Table 4), including the four main genera of lactic acid bacteria from fermented rice, such as *Latilactobacillus* sp., Limosilactobacillus, Weissella confusa, and *Pediococcus pentosaceus* (Table 4). The four isolates Lb-1, Lb-2, Lb-10, and Lb-11 showed similar sequences to *Latilactobacillus curvatus*, which was the dominant *Lactobacillus* sp. among the studied isolates. Other *Lactobacillus spp*., such as *Latilactobacillus graminis* and *Limosilactobacillus fermentum*, were also identified from the fermented rice.

3.3.4. Phytogenic Tree Development

The phylogenetic tree shows a relationship among the LAB species (Figures 5 and 6) isolated from fermented rice. The isolates of *Latilactobacillus curvatus* strains GRLb2, GRLb10, and GRLb11 show 98% homology among them. The *Pediococcus pentosaceus* strain GRLc1 shows 74% identity with *Latilactobacillus graminis* GRLb8. *Weissella confusa* strain GRLb4 shows a 99% phylogenetic relationship with *Latilactobacillus curvatus* strain GRLb1 and *Limosilactobacillus fermentum strain* GRLb17. *Latilactobacillus curvatus* strain GRLb1 shows 55% identity with *Limosilactobacillus fermentum strain* GRLb17. The microbial community in the phylogenetic tree is varied for different isolation sources. The phylogenetic tree of isolated microorganisms from fermented teff dough showed that *Lactobacillus* and *Enterococcus* sp. were the predominant microorganisms involved in the fermentation of teff dough [26]. The current study proved that the genera *Limosilactobacillus*, *Latilactobacillus*, *Weissella*, and *Pedicoccus* are the predominant microorganisms in fermented rice.



(**a**)

Figure 5. *Cont*.

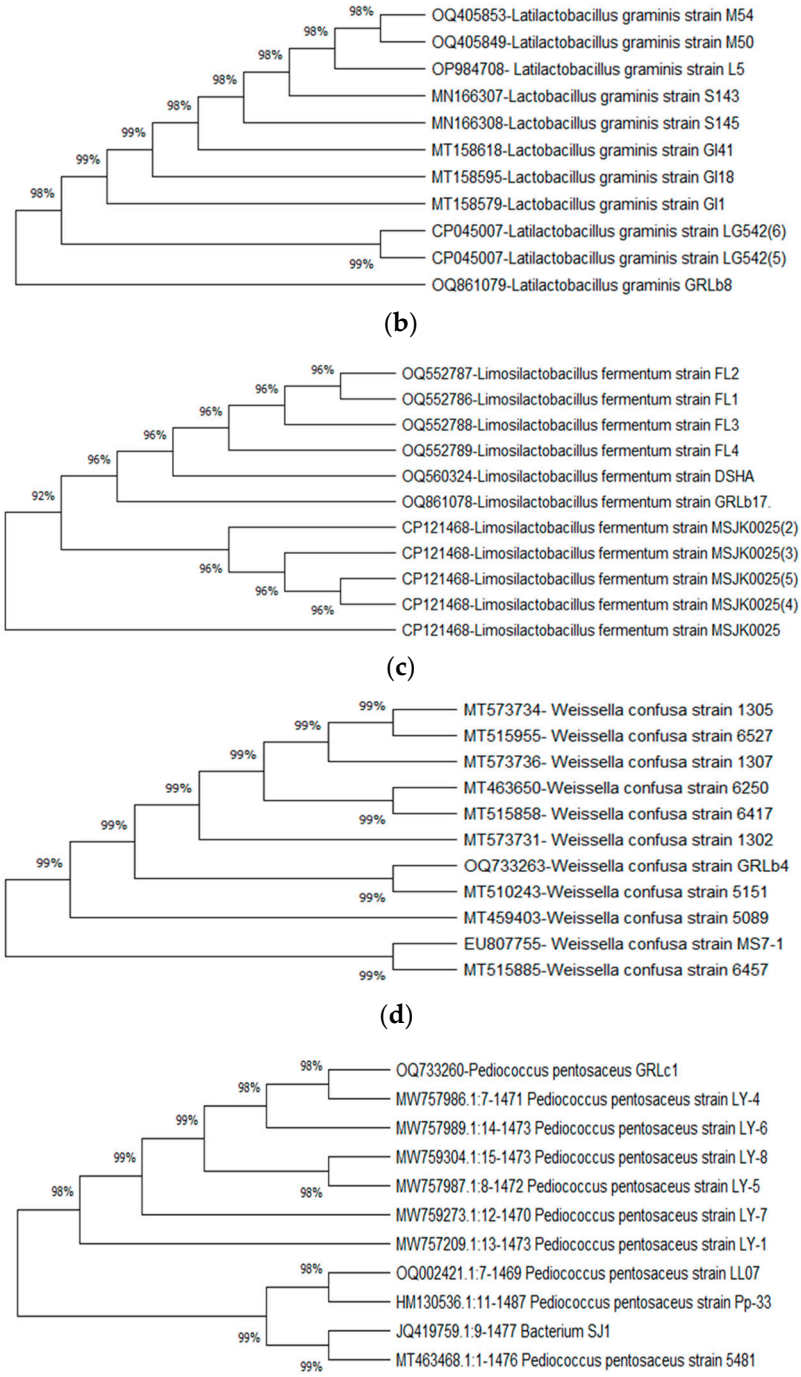


Figure 5. The phylogenetic trees (**a**–**e**) for each isolate (*Latilactobacillus curvatus* strains GRLb1, GRLb2, GRLb10, and GRLb11; *Latilactobacillus graminis* strain GRLb8; *Limosilactobacillus fermentum* strain GRLb17; *Weissella confusa* strain GRLb4; *Pediococcus pentosaceus* strain GRLc1), which were constructed using the neighbour-joining method in MEGA 11. The gene sequences of mostly related stains from Gene Bank (NCBI) were used to construct the phylogenetic trees.

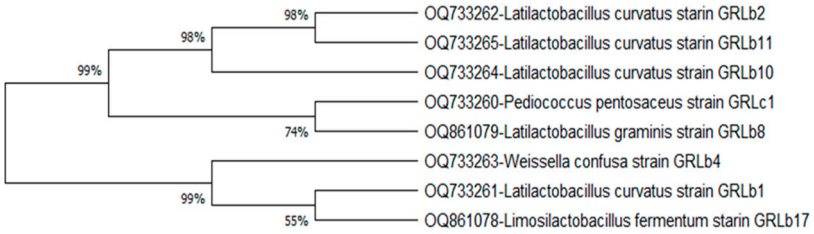


Figure 6. Phylogenetic tree of the isolates from fermented rice. The tree was constructed based on 16S rDNA gene sequences by using the neighbour-joining method in MEGA 11 software.

4. Discussion

Lactic acid bacteria (LAB) are well-known for their ability to synthesise a wide range of metabolites that beneficially affect the nutritional, sensorial, and technological properties of fermented food products. Among the studied isolates obtained from fermented rice, seven appeared as rods, and one was spherical in shape and found as a cluster. All isolates were Gram-positive and catalase-negative, and seven rod-shaped isolates were non-motile without spore formations. *Lactobacillus* appears as rod-shaped cells in short chains, while *Pediococcus* has spherical cells in pairs or tetrads. The morphological appearances of *Lactobacillus* and *Pediococcus* were rod and spherical, respectively. Seven rod-shaped isolates and one coccus could belong to the genera *Lactobacillus* and *Pediococcus*, respectively, as described by [27].

The phenotypic identification of microorganisms is important in the isolation and screening of lactic acid bacteria (LAB) with potential probiotic properties from new sources. *Lactobacillus* sp. is frequently found in vegetation, dairy products, and the human intestine, where it forms part of the normal flora of the gut [13]. The phenotypic characteristics of LAB can be used for identification at the genus level. The genus *Lactobacillus* can be confirmed using certain biochemical tests and Gram staining. *Lactobacillus* species are Gram-positive, catalase-negative, non-spore-forming, and non-motile rods (ranging from coccobacilli to long, slender bacilli) [28]. The physiological characteristics and fermentation of different types of sugars are used in the classification of *Lactobacillus* into groups; however, identification at species level is time-consuming, laborious, and difficult to interpret using conventional phenotypic methods [29]. The identification of *Lactobacillus at the* species level has been unreliable when only based on physiological, biochemical, and sugar fermentation tests [15]. The use of molecular-based techniques offers a rapid and species- or strain-specific alternative for identification [29].

The identification of Lactobacillus at the species level based on carbohydrate fermentation patterns using the API50 CHL kit is not a reliable method due to the similar nutrition requirements of lactobacilli. In a previous study of the carbohydrate fermentation pattern analysis of the two strains, *L. amylovorus* DSM20531 and *L. sobrius* DSM16698 were identified as *L. crispatus*, while *Lactobacillus reuteri* DSM 20016 was identified as *L. fermentum* [30]. PCR-based molecular techniques are precise and effective at identifying the species of lactic acid bacteria [31]. Species-specific PCR primers that target the 16S–23S rRNA spacer region are available for a limited number of Lactobacillus species. The 16S rRNA gene is used for phylogenetic studies, as it is highly conserved between different species of bacteria and archaea [16]. The accurate identification of Lactobacillus species can be accomplished by reference to 16S rRNA gene sequences and has been successful in the identification of different species of lactobacilli, including *L. plantarum*, *L. pentosus*, and *L. fermentum*, from different sources, such as the honey stomach of honeybees [32]. Furthermore, the Lactobacillus species, including *L. gastricus*, *L. santri*, *L. kalixensis*, and *L. ultunensis* isolated from human stomach mucosa [33]; *Lactobacillus plantarum, L. rhamnosus, and L. fermentum L. pentosus* from goat milk [15]; *L. nodensis* from rice bran [34]; *L. plantarum* and *L. fermentum* from fermented mustard (Susilini et al. [17]); *L. saerimneri* from pig feces [35]; and *L. casei* NRC AM2, L. s *rhamnosus* NRC AM6, *P. pentosaceus* NRC AM4, and *P. acidilactici* NRC AM8 from fermented dairy products [31] have been successfully identified at the molecular level

using 16S rRNA gene sequences. The molecular identification method is more accurate and confirmative for the identification of *Lactobacillus* sp. compared with other biochemical identification methods [36]. Therefore, the isolated *Lactobacillus* sp. was confirmed using 16S rDNA gene sequencing.

In the current study, isolates Lb-4, Lb-8, Lb-10, and Lb-17 were not identified as similar species by biochemical identification using API 50 kits and the 16S rDNA method. Isolates Lb-1, 2, 11, and Lc-1 were identified as similar species to *Lactobacillus curvatus* and *Pediococcus pentosaceus* by both identification methods. Lactic acid bacteria belonging to the genus Lactobacillus were identified as *L. curvatus*, *L. delbrueckii*, *L. helveticus*, *L. pentosus*, *L. plantarum*, and *Pediococcus pentoceous* using the API 50 CHL system. While molecular identification by 16S rDNA confirmed the five lactic acid bacteria as *Weissella confusa*, *Pediococcus pentosaceus*, *Latilactobacillus curvatus*, *Latilactobacillus graminis*, and *Limosilactobacillus fermentum* in fermented rice, *Latilactobacillus curvatus* was the dominant *Lactobacillus* sp. in fermented rice (Tables 3 and 4). Homemade fermented rice is traditionally prepared by soaking the cooked or raw rice in water in a natural environment, which was traditionally consumed by the Asian farming community and is largely ignored in modern life. The wild bacteria present in the air or rice ferment the rice. The microorganisms responsible for the fermentation of naturally fermented food have not been well studied in the past; the current study confirmed the five species of lactic acid bacteria that are involved in the fermentation of traditionally fermented food in Asia. Further clinical studies will confirm the health benefits of fermented rice.

Previous studies show that similar species isolated from fermented foods have probiotic characteristics. Twenty-five strains of lactic acid bacteria (LAB) have been isolated from South Indian traditional fermented foods, including 'Kallappam' batter, 'Koozh', and 'Mor Kuzhambu'. Six strains were identified with higher antimicrobial activity among the twenty-five strains [37]. *Lactobacillus fermentum*, which was isolated from Thai traditional fermented food products (fermented pork, fermented fish, fermented tea leaves, and pickled garlic), showed extremely high survival rates in acid or bile salts, inhibited pathogenic bacteria, and was sensitive to antibiotics [38]. *Lactobacillus* species isolated from fermented rice showed antimicrobial activity against pathogenic bacteria, including *Salmonella thyphi*, *Salmonella enteritidis*, *Salmonella thyphimurium*, *Escherichia coli* (ATCC 259222), *Shigella sonnei*, *Shigella flexneri*, and *Candida spp.* [39]. *Lactobacillus curvatus* A61 inhibits the growth of *Listeria monocytogenes* and *Bacillus cereus* strains and has shown antifungal activity against *Cladosporium* and *Fusarium* sp. [40]. *Weissella confusa* C3-7 shows antifungal activity against *Penicillium roqueforti*, *Aspergillus niger*, and *Endomyces fibuliger* in contaminated bakery products [41]. *Pediococcus pentoceus* was the dominant isolate found in fermented cooked white rice and cocoa milk [42]. The strains of *Pediococcus pentosaceus* (MY-800, NS75, KC007, and SH 740) isolated from Indian fermented food possessed probiotic characteristics [43]. *L. fermentum* FTL2311 and *L. fermentum* FTL10BR isolated from fermented tea leaves were probiotics with satisfactory acid and bile tolerance and antimicrobial activity [38]. *Lactobacillus curvatus* HY7602-added fermented antler (FA) is a functional food that effectively controls muscle atrophy caused by aging and is considered a novel alternative treatment for sarcopenia [44]. The review study by Ying Chen et al. [45] shows that *Latilactobacillus curvatus* is a probiotic with excellent fermentation properties and offers health benefits. LAB isolated from 'idli' batter [46] and *Lactobacillus* species isolated from fermented buffalo milk gel have demonstrated promising probiotic potential with antibiotic sensitivity, antimicrobial properties, bile acid tolerance, and acid tolerance [47]. As per the above review, the similar species of studied isolates were identified as probiotics and used as starter cultures for the production of fermented food. Thus, the microbial community from fermented rice may possess probiotic properties, and fermented food may be considered a functional food. The probiotic characterisation of the species identified in the present study warrants further studies.

5. Conclusions

Five species of lactic acid bacteria, including *L. curvatus*, *L. graminis*, *L. fermentum*, *W. confusa*, and *P. pentosaceus,* were identified using 16S rDNA molecular technology. *L. curvatus* was the dominant *Lactobacillus* sp. among the studied isolates from fermented rice. Most of the isolates showed homofermentative characteristics except for the isolate Lb-8, which showed heterofermentation. Isolates were able to grow at temperatures ranging between 10 °C and 45 °C, tolerate NaCl concentrations from 2% to 6.5, and coagulate the milk. Fermented rice may offer health benefits due to the presence of LAB. The favourable physiological characteristics of these isolates confirm their potential application in the food industry. However, further studies are required to confirm the probiotic properties of these isolates, their specific biochemical reactions and secondary metabolite production during fermentations, and their health benefits to humans.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9090807/s1.

Author Contributions: Conceptualization, N.J.; data curation, N.J. and T.M; formal analysis, N.J.; investigation, N.J.; software, N.J.; methodology, N.J.; validation, N.J.; writing—original draft preparation, N.J.; writing—review and editing, C.S.R., M.Y.M., A.G., O.M., and T.M.; supervision, C.S.R., M.Y.M., and T.M.; resources, C.S.R., M.Y.M., and T.M. All authors have read and agreed to the published version of the manuscript.

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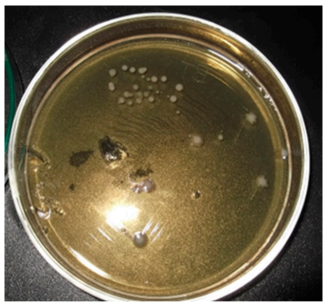
Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Appendix A



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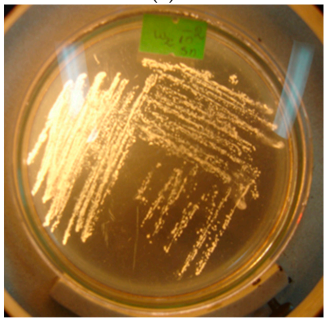

(**b**)

Figure A1. Isolated colonies of Lb-8 (**a**) and cluster-like cocci (**b**) from fermented rice.

REFERENCE **DATE**
1 8/8/13
COMMENT
48 incubation-1

GOOD IDENTIFICATION	
Strip	API 50 CHL V5.1
Profile	-----+----++++--------+--++-+--------------------
Note	

Significant taxa	% ID	T	Tests against		
Lactobacillus curvatus ssp curvatus	98.9	0.98			

Next taxon	% ID	T	Tests against			
Lactobacillus plantarum 2	0.5	0.7	MAN80%	AMY83%	LAC75%	GEN83%

Figure A2. Identification of Lactobacillus using API web.

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