Phenotypic and genotypic characterisation of *Lactobacillus* and yeast isolates from a traditional New Zealand Māori potato starter culture

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ABSTRACT

Parāroa Rēwena is a traditional Māori sourdough produced by fermentation using a potato starter culture. The microbial composition of the starter culture is not well characterised, despite the long history of this product. The morphological, physiological, biochemical and genetic tests were conducted to characterise 26 lactic acid bacteria (LAB) and 15 yeast isolates from a Parāroa Rēwena potato starter culture. The results of sugar fermentation tests, API 50 CHL tests, and API ID 32 C tests suggest the presence of four different LAB phenotypes and five different yeast phenotypes. 16S rRNA and 26S rRNA sequencing identified the LAB as *Lacticaseibacillus paracasei* and the yeast isolates as *Saccharomyces cerevisiae*, respectively. Multilocus sequence typing (MLST) of the *L. paracasei* isolates indicated that they had identical genotypes at the MLST loci, to *L. paracasei* subsp. *paracasei* IBB 3423 or *L. paracasei* subsp. *paracasei* F19. This study provides new insights into the microbial composition of the traditional sourdough Parāroa Rēwena starter culture.

1. Introduction

Sourdough fermentation using natural starter cultures is one of the oldest biotechnological processes used for leavened bread production. Globally, a number of traditional baked products, such as French breads, Italian breads and soda crackers are produced using sourdough fermentation (Clarke and Arendt, 2005). Sourdough fermentation improves acidification, flavour, texture, and shelf life of the final bread product due to symbiotic fermentation by the microorganisms in the starter culture. Sourdough is composed mainly of flour and water fermented by lactic acid bacteria (LAB) and yeast (Aplevicz et al., 2013). The production of lactic acid, acetic acid, ethanol, and CO_2 is responsible for dough-leavening and the development of the desirable slightly sour taste of the product, which is dependent on the specific types of fermenting LAB and yeast present in the starter culture (Cauvain and Young, 2007).

In New Zealand, indigenous Māori have baked and consumed traditional Rēwena sourdough bread for more than a century (Rush et al., 2010). The current traditional practice of making Rēwena bread uses back-slopping fermentation with a potato starter culture (PSC), with some back-slopped cultures having been maintained for many decades (Albala, 2011; McLean, 2004). As with other sourdoughs, Rēwena PSC may contain both LAB and yeast, but the specific microbial composition of the potato culture has not been documented. Furthermore, different PSCs are likely to have diverged in composition during sequential fermentations. The lack of knowledge about the types and range of microbial communities in PSCs makes it difficult to control the fermentation process of Rēwena sourdough breadmaking.

LAB are classified either as obligate homo-fermentative, facultative hetero-fermentative, or obligate hetero-fermentative LAB. The LAB in sourdough, especially *Lactobacillus* species, contribute to the acidification and dough development during sourdough fermentation (Chavan and Chavan, 2011). Homo-fermentative LAB mainly produce lactic acid from glucose through the glycolytic pathway, while hetero-fermentative LAB mainly produce lactic acid, acetic acid, CO_2, and ethanol via the 6-phosphogluconate pathway. The production of ethanol depends on the presence of acetaldehyde, which acts as an electron acceptor (Axelsson, 2004; Corsetti and Settanni, 2007). Nearly 50 different species of LAB

have been isolated from sourdough (Arendt et al., 2007; De Vuyst and Neysens, 2005), including a wide range of species from the genus *Lactobacillus*, as well as species from other genera such as *Leuconostoc*, *Pediococcus*, *Weissella* and *Enterococcus* (Arendt et al., 2007). Common *Lactobacillus* isolated from traditional sourdough include *L. brevis, L. fermentum, L. paralimentarius, L. plantarum, L. pontis* and *L. sanfranciscensis* (Nionelli and Rizzello, 2016).

In addition to LAB, yeast are usually found in sourdough, and the ratio of yeast cells to LAB cells generally ranges from 1:10 to 1:100 (Paterson and Piggott, 2006). The primary function of yeast is leavening, which is caused by the production of CO_2. In sourdough, yeast compete with LAB for carbohydrate sources (primarily maltose), while withstanding the stresses of low pH and low oxygen (Minervini et al., 2014). More than 20 species of yeast have been found in sourdough, with *Saccharomyces cerevisiae* being the dominant species (Rossi, 1996), although *Saccharomyces exiguous, Candida humilis, Issatchenkia orientalis (Candida krusei), Pichia anomala, Saturnispora aitoi (Pichia saitoi), Torulaspora delbrueckii, Debaryomyces hansenii*, and *Pichia membranifaciens* have also been reported (Chavan and Chavan, 2011).

To date, no studies have been carried out on the microbial composition of the PSC used in making the traditional New Zealand Māori Rēwena bread. Therefore, the aim of the current study was to isolate, characterise, and identify LAB and yeast in the PSC, based on a combination of physiological, biochemical, and genetic assays of LAB and yeast cells isolated from the PSC.

The strains were also characterised using a genetic approach. Although genetic classifications are usually viewed as being gold-standard, there is no guarantee that the genetic classification is indicative of the phenotypic status of the isolates. Therefore, the present study also aimed to investigate whether the phenotypic characteristics of the isolated microorganisms follow those expected based on the genetic classification.

2. Materials and methods

2.1. Source of potato starter culture

An undefined mixed liquid starter culture contained boiled mashed potatoes (Solanum tuberosum L.), high grade flour, water recovered from boiled potatoes, refined sugar) was supplied by Māori Kai Cuisine Ltd (Tauranga, New Zealand) and transported at ambient temperature (20 °C) to Massey University Laboratories (Auckland, New Zealand). The potato starter culture was analysed immediately upon receipt. Of the starter culture, 10 g were aseptically withdrawn, and suitable serial dilutions were prepared for plating on suitable medium. The remainder was stored frozen at −80 °C.

2.2. Microbiological analysis of the potato starter culture

2.2.1. Enumeration and propagation of microorganisms

The enumeration and propagation of LAB and yeast in the PSC was performed by plating serial diluted samples on suitable media according to previous reports (Meroth et al., 2003) with minor modifications. LAB were plated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid, UK). Cycloheximide (0.01%) (Sigma Aldrich, USA) was added to MRS agar plates to inhibit the growth of yeast. MRS agar was chosen based on its successful use in several studies that successfully isolated LAB from different sourdoughs (De Vuyst and Vancanneyt, 2007; Hassan and Bullerman, 2008; Karaman et al., 2018; Sáez et al., 2017). Yeast were plated on Yeast Extract Glucose Chloramphenicol (YGC) agar (Merck, Germany) (Häggman and Salovaara, 2008; Meroth et al., 2004).

To obtain single colonies, 10 g of starter culture was measured into a sterile stomacher bag (VWR-Global Science) and homogenised with 90 g peptone water (Merck, Germany) for 2 min in a stomacher laboratory paddle blender (IUL instruments, Spain). Ten-fold serial dilutions were plated on MRS, and YGC agar plates, followed by incubation (Liu et al.,

Table 1
Incubation conditions for inoculated MRS agar and YGC agar plates.

Type of Agar	Incubation temperature (°C)	Incubation time (h)	Incubation environment
MRS	37	48 ± 2	Anaerobic
YGC	25	120 ± 2	Aerobic

2015; Mithun et al., 2015) (Table 1). After growth, colonies were examined for shape, elevation and margin, surface, colour, and size (Bennani, Mchiouer, Rokni and Meziane, 2017a). Morphologically distinct colonies were selected and purified as described in subsequent sections.

2.2.2. Isolation and purification of colonies

Representative LAB and yeast colonies with distinct morphologies were purified by streaking on suitable agar plates. All cells were confirmed as being Gram positive by Gram staining and examination under oil immersion using the Carl Zeiss transmission light microscope (Model HBO 50/AC, Germany) at 100× magnification. Cultures of purified colonies were preserved in 80% glycerol (Sigma Aldrich, USA) for long term storage at −80 °C.

2.3. Characterisation of LAB and yeast

2.3.1. Catalase test for LAB

Catalase testing was carried out according to the method of Kaban and Kaya (2008) with some minor modifications. LAB cultures streaked on MRS agar plates were grown for 48 h, then specimens from the colonies were transferred onto a glass slide with a sterile loop followed by addition of 1–2 drops of freshly prepared 3% hydrogen peroxide. The mixture on the glass slide was examined immediately for formation of bubbles, which is indicative of a positive catalase reaction. *Lactobacillus* are considered as catalase negative, therefore, any cultures with catalase negative reactions were subjected to further physiological and biochemical tests. All tests were conducted in triplicate.

2.3.2. Growth of LAB at different temperatures

The growth of LAB at different temperatures was carried out according to the methods of Kaban and Kaya (2008) with minor modifications. Each tested isolate was inoculated in 5 mL of MRS broth and incubated anaerobically at 37 °C overnight. From the overnight grown culture, 50 μL was inoculated into 5 mL of MRS broth and incubated anaerobically at 15 °C and 45 °C for 7 days and then examined for turbidity. All tests were conducted in triplicate.

2.3.3. Acid and gas production by LAB from glucose metabolism

The test for acid and gas production during glucose metabolism by lactic acid bacteria was performed as described by Ali (2011) with minor modifications. The medium used was prepared by dissolving 6.5 g nutrient broth (Himedia, India), 2.5 g glucose (Ajax Finechem, Australia) and 0.009 g phenol red indicator (Difco Laboratories Inc, USA) in 500 mL distilled water. An overnight activated culture (60 μL) was inoculated into 6 mL of the medium with an inverted Durham tube and incubated anaerobically at 37 °C for 48 h. Samples were examined for gas production and change of colour. All tests were conducted in triplicate.

2.3.4. Ammonia production by LAB from arginine

The test for ammonia production was carried out according to the method of Harrigan (1998). The test medium used was prepared by dissolving 6.5 g nutrient broth (Himedia, India), 0.5 g glucose (Ajax Finechem, Australia), 0.008 g bromocresol purple indicator (Sigma Aldrich, USA), 2.5 g L-Arginine monosaccharides (Sigma Aldrich, USA) in 500 mL distilled water and the mixture was autoclaved. An overnight activated culture (50 μL) was inoculated into 5 mL test medium and

incubated anaerobically at 37 °C for 48 h. Three-four drops of Nessler's reagent (Sigma Aldrich, USA) were added to the test tubes and examined for colour change. All tests were conducted in triplicate.

2.3.5. Carbohydrate fermentation by LAB

Ten sugars were tested for carbohydrate fermentation by LAB isolates using the API 50 CHL medium (bioMe'rieux, Inc., Marcy I'Etolie, France). The carbohydrates used were lactose (Ajax Finechem, Australia), trehalose (Sigma Aldrich, USA), sucrose (Sigma Aldrich, USA), melibiose (Sigma Aldrich, USA), ribose (GF Biochemicals, America), sorbitol (Sigma Aldrich, USA), mannitol (Sigma Aldrich, USA), melezitose (Sigma Aldrich, USA), galactose (Sigma Aldrich, USA) and xylose (Fort Richard, Auckland). Each isolate was inoculated in 5 mL MRS broth and incubated anaerobically overnight to activate the culture. Activated cells were streaked onto MRS agar plates and incubated at 37 °C for 24 h. Bacterial cells (18–24 h old) that grew on the MRS agar plate were inoculated into 2 mL fresh distilled water until a turbidity of 0.451 at 600 nm was achieved, which was approximately 6.0×10^8 cells/ml of the bacterial suspension. From the mixed sample, 50 μL was added to 100 μL of medium solution in 96 well plates. The plates were incubated anaerobically at 37 °C for 24–48 h and examined for purple to yellow colour change. During LAB fermentation of sugars, acids are produced in the medium which becomes acidic resulting in the change of colour. All tests were conducted in triplicate.

The API 50 CHL kit (bioMe'rieux, Inc., Marcy I'Etolie, France) was used to characterise for the genus *Lactobacillus* based on the metabolism of specific sugars during fermentation according to the manufacturer's instructions. Inoculated strips were incubated at 37 °C for 24–48 h and then examined for a colour change from purple to yellow (reduction in pH) which is indicative of a positive result. Results were recorded and interpreted by apiweb™ identification software database (https://apiweb.biomerieux.com). Different LAB oxidise different sugars to produce adenosine triphosphate (ATP) during sugar metabolism using inherent enzymes (Khalid, 2011).

2.3.6. API ID 32 C test for yeast

The API ID 32 C kit (bioMe'rieux, Inc., Marcy I'Etolie, France) was used to characterise yeast-based carbohydrate assimilation (Ramani et al., 1998). The API ID 32 C test for yeast was conducted according to the manufacturer's instructions. The incubated test cupules were compared to the control (cupule) and examined for development of turbidity. Results were recorded and interpreted using the apiweb™ identification software database (https://apiweb.biomerieux.com).

2.4. Extraction of genomic DNA from isolated LAB colonies

The isolation of genomic DNA from LAB isolates was carried out according to the method of Munoz-Quezada et al. (2013) with some modifications. Briefly, one loopful of purified LAB isolate was inoculated into 10 mL MRS broth and incubated at 37 °C overnight. Ten microliters of mixed activated LAB cell suspension were transferred into 10 mL of fresh MRS broth and incubated at 37 °C for 2 h. The cell pellet was harvested by centrifugation at 5500*g* (Heraeus Biofuge PrimoR, Thermo Fisher, Germany) for 10 min and washed three times with 1 mL buffer A (30 mM Tris-HCl (pH 8.0) (Ajax Finechem, Australia), 5 mM EDTA (BDH Chemicals Limited, England) and 50 mM NaCl (Sigma Aldrich, USA). The washed cell pellet was resuspended in 1 mL of buffer B (30 mM Tris-HCl (pH 8.0), 5 mM EDTA, 50 mM NaCl, 25% (w/v) sucrose (Univar, UK), 20 mg/mL lysozyme (Thermo Fisher, Germany) and 20 μL mutanolysin (Thermo Fisher, Germany) and incubated at 37 °C for 1 h to degrade cell walls (Srivastava et al., 2018). An additional 0.5 mL of 0.25 M EDTA was added into the cell suspension and incubated at 22 °C for 5 min to inhibit the activity of the DNase enzyme. Fifty microliters of 20% (w/v) Sodium Dodecyl Sulphate (SDS; J. T. Baker, USA) was added to the cell suspension and incubated at 65 °C for 30 min to denature cell membrane proteins. Next, 10 μL of proteinase K (20 mg/mL) (Qiagen,

Table 2
List of MLST primers.

Primer	Sequence
ftsZ_F	GGCATTGCACAACTGAAAGA
ftsZ_R	GCATCGTCTGCGTTAGTTTG
polA_F	TTATCATGTGGCCGAACAAA
polA_R	GTTTGCGTCAAAGTCTGCAA
mutL_F	ATCGGCAACATTAAGCAACC
mutL_R	GATGACGCCCATTGGATAAC
metRS_F	CGGTATTTTGCCAGCCTTTA
metRS_R	CATTTCGCCTTTTAGCTTGC
nrdD_F	GCTTGAAGCGTGATTTAGCC
nrdD_R	ACATTCGATCGCCAATTGTT
pgm_F	AGGCATTTGCTGCTCCTATG
pgm_R	GGGATCAGTCGCGATTAAGA

Germany) was added and the mixture incubated at 65 °C for 30 min to degrade proteins and lyse the cells. An equal volume (1.6 mL) of phenol-chloroform-isoamyl alcohol solution (25:24:1) was added to the cell lysate, mixed thoroughly and centrifuged at 8000 *g* for 10 min. The aqueous (top) layer containing the DNA was carefully transferred to a new tube and the extraction steps repeated three times until no visible white layer was observed. Ten microliters of DNase-free RNase A (100 μg/mL; Sigma Aldrich, USA) was added to the DNA solution and incubated at 37 °C for 1 h to remove any remaining RNA. The DNA was then precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2; Thermo Fisher Scientific, UK) and 2.5 volume of 95% (v/v) cold ethanol, followed by centrifugation at 14000*g* for 10 min. The DNA pellet was washed in 70% (v/v) ethanol and centrifuged at 8000 *g* for 10 s. Finally, the DNA pellet was air-dried and resuspended in 50 μl 10 mM Tris-HCl (pH 8) and stored at −20 °C for further analysis.

2.4.1. 16S rRNA PCR reactions

The 16S rRNA region was amplified by PCR using the universal primers 27F (5′- AGAGTTTGATCMTGGCTCAG- 3′) and 1492R (5′-GGTTACCTTGTTACGACTT -3′), as reported by Liu et al. (2018) and were supplied by Invitrogen (Thermo Fisher Scientific, New Zealand). Cycling conditions were: (1) initial denaturation at 94 °C for 2 min; (2) 30 cycles of: 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 1 min; and (3) the final extension at 72 °C for 5 min. The PCR amplified DNA fragments were analysed by gel electrophoresis.

2.4.2. 16S rRNA sequencing of PCR products of extracted DNA from isolated LAB colonies

16S rRNA Sanger sequencing was performed by Macrogen Inc. (Seoul, Korea) using the 785F (5′- GGATTAGATACCCTGGTA -3′) and 907R (5′- CCGTCAATTCMTTTRAGTTT -3′) primers. Species were identified using BLAST. An identity of over 99.9% was considered as the same species.

2.4.3. 26S rRNA sequencing of isolated yeast colonies

Five isolated yeast colonies on agar plate were randomly picked and sent to Macrogen Inc., Seoul, Korea for 26S sequencing analysis following identification by the API ID 32 C test. The forward primer LR0R (5′- ACCCGCTGAACTTAAGC -3′) and reverse primer LR7 (5′-TACTACCACCAAGATCT -3′) primers were used for both PCR and Sanger sequencing. Species were identified using BLAST. An identity of over 99.9% was considered to be the same species.

2.4.4. PCR of MLST loci

LAB isolates were streaked on MRS agar plates with anaerobic incubation at 37 °C for 24 h. Three single colonies were picked and transferred into separate tubes, each containing 100 μL of sterile distilled water. The tubes were heated at 95 °C for 5 min. Each of the heated colony suspensions was used as a template for multiplex PCR, using primer pools amplifying six loci on the *Lactobacillus* chromosome: *ftsz*, *mutL*, *metRS*, *nrdD*, *pgm*, and *polA* (Table 2) (Cai et al., 2007). For

Table 3
Morphology of LAB colonies grown on MRS agar plates.

Colony Type[a]	Diameter (mm)	Shape	Margin
1	1.5–2	Circular	Entire
2	2–3	Circular	Undulate
3	5–8	Irregular	Undulate

[a] Colonies were characterised by observing their size, shape, margin, elevation, surface, and colour.

multiplex PCR, all primers were pooled in equimolar amounts to make a stock solution with 10 mM concentration, and a concentration of 1 mM was used in the PCR reaction. NEB Q5 High-Fidelity polymerase was used for multiplex PCR. Cycling conditions were as follows: initial denaturation at 95 °C for 2 min; 35 cycles of: 95 °C for 20 s, annealing at 53 °C for 30 s, and extension at 72 °C for 5 min; followed by a final extension at 72 °C for 5 min.

2.4.5. MLST sequencing via Oxford Nanopore

The multiplex PCR reactions were carried forward for Oxford Nanopore library preparation using the Oxford Nanopore Rapid Barcode kit (RBK-004) according to the manufacturer's instructions. The library was loaded onto a single MinION flow cell. The three samples were multiplexed on three barcodes with 12 additional unrelated samples. The concentration of the MLST products was approximately 50-fold less than the concentration of the unrelated samples, resulting in more than 50-fold fewer reads relative to the other samples. The flow cell was run for 1 h 47 m, resulting in a total of 531, 666, and 345 reads for the three LAB samples, with an average length of 519 bp, 515 bp, and 496 bp for each, respectively. No reads were obtained for the negative control samples.

2.4.6. Determination of consensus sequences from Oxford Nanopore data

The Oxford Nanopore sequence reads were trimmed with Porechop (Wick et al., 2017) and mapped onto a set of reference sequences consisting of *ftsz*, *mutL*, *metRS*, *nrdD*, *pgm*, and *polA* loci from *Lactobacillus paracasei* JCM 8130 using minimap2 (Li, 2018). The resulting bam files were filtered for mapped reads using SAMtools (Li et al., 2009). Fastq files for the mapped reads were then obtained using the SAMtools

bam2fastq function. This yielded a median of 78 mapped reads per locus, with a minimum of 21 reads and a maximum of 197 (IQR 59–104).

Medaka (https://github.com/nanoporetech/medaka) was used to produce new consensus sequences based on sequence correction of the *Lactobacillus paracasei* JCM 8130 loci. All consensus sequences were confirmed using Sanger sequencing of single-locus PCR products, with reactions performed in both the forward and reverse directions. All were identical to the Medaka consensus sequences. https://github.com/osilander/flo-mlst.

2.4.7. Phylogenetic analysis

To obtain MLST loci from other *Lactobacillus*, a BLAST search of the NCBI nt database was performed using each of the *Lactobacillus paracasei* JCM 8130 MLST sequences. All hits with a length of at least 99% of the query sequence were retained. All sequences for each locus were aligned using MAFFT v7.471 with the adjust direction option (Katoh and Standley, 2013). This alignment was used as input to IQTree v2.03. The phylogeny was obtained using a Kimura 2-parameter substitution matrix with two categories of rate variation across sites (determined by ModelFinder to be the best fit model). One thousand ultrafast bootstraps were performed to obtained bootstrap values (Huson and Bryant, 2006).

3. Results and discussion

3.1. Microbiological analysis of potato starter culture

The original potato starter culture contained 1.58×10^9 CFU/mL of LAB cells and 3.8×10^7 CFU/mL of yeast cells. These results are similar to those reported for other sourdough microbiota (De Vuyst and Neysens, 2005; De Vuyst et al., 2014). As the cell density exceeded 10^8 CFU/g in the sourdough, it was considered fully fermented (De Vuyst and Neysens, 2005). There were 26 isolated LAB colonies and observed three different morphological types (Table 3). The divergent morphologies suggested that the LAB may belong to different species or strains (Bennani, Mchiouer, Rokni and Meziane, 2017b). All of the LAB colonies appeared flat, smooth, and opaque. The diameters observed for LAB type 1 and type 2 colonies varied from 1.5 to 3 mm, consistent with *L. casei*

Table 4
Sugar metabolism of 26 LAB isolated from sourdough potato starter culture.

LAB isolate		Gas from glucose	Melibiose	Xylose	Sucrose	Mannitol	Sorbitol	Lactose	Melezitose	Galactose	Ribose	Trehalose
Group 1	4-3A1	–	–	–	+	+	+	+	+	+	+	+
	4-4A1	±	–	–	+	+	+	+	+	+	+	+
	4-4A2	–	–	–	+	+	+	+	+	+	+	+
	4-4A3	–	–	–	+	+	+	+	+	+	+	+
	4-4B1	–	–	–	+	+	+	+	+	+	+	+
	4–5A2	–	–	–	+	+	+	+	+	+	+	+
	4–5A3	–	–	–	+	+	+	+	+	+	+	+
	4–5A4	–	–	–	+	+	+	+	+	+	+	+
	4–5A5	–	–	–	+	+	+	+	+	+	+	+
	4–5A6	–	–	–	+	+	+	+	+	+	+	+
	4–5A8	–	–	–	+	+	+	+	+	+	+	+
	4–6A1	–	–	–	+	+	+	+	+	+	+	+
	4–6A2	–	–	–	+	+	+	+	+	+	+	+
	4–6B1	–	–	–	+	+	+	+	+	+	+	+
	4–6B2	–	–	–	+	+	+	+	+	+	+	+
	80-4A1	±	–	–	+	+	+	+	+	+	+	+
	80-4A2	±	–	–	+	+	+	+	+	+	+	+
	80-4A3	–	–	–	+	+	+	+	+	+	+	+
	80-5B2	–	–	–	+	+	+	+	+	+	+	+
	80-6B1	±	–	–	+	+	+	+	+	+	+	+
Group 2	4–7B1,2	–	±	–	+	+	+	+	+	+	+	+
	4–7B3	±	±	–	+	+	+	+	+	+	+	+
	80-5B1	±	±	–	+	+	+	+	+	+	+	+
Group 3	80-5A3	±	–	±	+	+	+	+	+	+	+	+
Group 4	80-5B3	±	±	±	+	+	+	+	+	+	+	+
	80-5A1	–	±	±	+	+	+	+	+	+	+	+

Note: + = positive; - = negative; ± = positive/negative.

Table 5
Carbohydrate metabolism of four representative LAB isolates.

Carbohydrate	Isolate #			
	4-4A1	4–7B1,2	80-5B3	80-5A3
Negative Control	–	–	–	–
Dulcitol	–	–	+	+
Raffinose	–	–	+	+
Rhamnose	–	–	+	+
2-keto-gluconate	–	–	–	–
5-keto-gluconate	–	–	–	–
D-Arabinose	–	–	–	–
D-arabitol	–	–	–	–
D-fucose	–	–	–	–
D-lyxose	–	–	–	–
D-xylose	–	–	±	±
Erythritol	–	–	–	–
Glycerol	–	–	–	–
Glycogen	–	–	–	–
Inositol	–	–	–	–
Inulin	–	–	–	–
L-Arabinose	–	–	–	–
L-arabiotl	–	–	–	–
L-fucose	–	–	–	–
L-xylose	–	–	±	±
Melibiose	–	±	±	–
Starch	–	–	–	–
Xylitol	–	–	–	–
B-methyl-D-xyloside	–	–	–	–
Adonitol	+	+	+	+
Amygdalin	+	+	+	+
Arbutin	+	+	+	+
Celibiose	+	+	+	+
D-tagatose	+	+	+	+
Esculin	+	+	+	+
Fructose	+	+	+	+
Galactose	+	+	+	+
Gentibiose	+	+	+	+
Gluconate	+	+	+	+
Glucose	+	+	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Mannitol	+	+	+	+
Mannose	+	+	+	+
Melezitose	+	+	+	+
N-acetyl-glucosamine	+	+	+	+
Ribose	+	+	+	+
Salicin	+	+	+	+
Sorbitol	+	+	+	+
Sorbose	+	+	+	+
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Turanose	+	+	+	+
A-methyl-D-glucoside	+	+	+	+
A-methyl-D-mannoside	+	+	+	+
Presumptive species (% Identity)	*L. paracasei* spp. *paracasei* 1 (99.1%)	*L. paracasei* spp. *paracasei* 1 (99.1%)	*L. rhamnosus* (99.9%)	*L. rhamnosus* (99.9%)

Note: + = positive result; - = negative result; ± = positive/negative; Results were analysed by apiweb™

and *L. paracasei* species, which range from 2 to 5 mm and 1–3 mm, respectively. (Sutula et al., 2012). Type 3 colonies exhibited undulate margins of larger size than the other types. This may be due to an accumulation of smaller colonies with entire margins, or large colonies with undulate margins.

There were 15 yeast isolates obtained, all of which were flat and opaque with smooth surfaces when plated on YGC agar plates. The

diameters of the colonies were 2–9 mm, and hence within the expected range for *Saccharomyces cerevisiae* (Clemons et al., 1996). The yeast cells had a diameter of 4–5 μm, consistent with that of commercial yeast, *S. cerevisiae* (Aon et al., 2018).

3.2. Characterisation of LAB and yeast isolates

All 26 LAB isolates were Gram positive, catalase negative, and were able to grow at 15 °C and 45 °C, which was consistent with the characteristics of *Lactobacillus* (Khedid et al., 2009). Table 4 shows the results of gas production from glucose, utilisation of melibiose and xylose by the LAB isolates. Of all the tested LAB isolates utilised glucose (Table 4), a characteristic common to all *Lactobacillus* sp. (Nikita and Hemangi, 2012). *Lactobacillus* sp. can be classified into obligate homofermentative, facultative heterofermentative and obligate heterofermentative. Only obligate heterofermentative *Lactobacillus* can ferment glucose to produce gas (Giraffa, 2014; Holzapfel and Wood, 2014). The LAB isolates in this study were able to hydrolyse arginine to produce ammonia, which is a typical feature of most heterofermentative *Lactobacillus*. Based on these results, the presumptive LAB isolates appeared to be heterofermentative or homofermentative *Lactobacillus*.

Different *Lactobacillus* species metabolise different carbohydrates as energy sources, and the pattern of carbohydrate utilisation can be used to determine the probable species of isolates (Puniya et al., 2012). Therefore, the metabolism of trehalose, melibiose, sucrose, mannitol, sorbitol, lactose, melezitose, galactose, ribose and xylose were tested by all LAB isolates. All LAB isolates were able to metabolise all sugars except for melibiose and xylose (Table 4). Comparison of these results with published data on *Lactobacillus* (Vos et al., 2011) indicated that all isolates were likely to be either *L. paracasei* subsp. *paracasei* or *L. rhamnosus*.

Based on the differential carbohydrate metabolism patterns, the LAB isolates were classified into four groups, and one isolate from each group was selected for API 50 CHL testing (Table 5). The results indicated that the reiterative four tested LAB isolates from the 26 LAB isolates were most likely to be L. paracasei subsp. paracasei or L. rhamnosus. The same carbohydrate fermentation patterns were obtained for each of the four LAB colonies except for rhamnose, dulcitol, and raffinose. Only colonies 80-5B3 and 80-5A3 were able to ferment rhamnose, dulcitol, and raffinose, while colonies 4-4A1 and 4–7B1,2 were not able to utilise these three sugars as an energy source. According to previous studies, L. rhamnosus ferment rhamnose and dulcitol, while L. paracasei subsp. paracasei are not able to ferment either of the two sugars. Neither of the two species can ferment raffinose (Hedberg et al., 2008; Koryszewska-Baginśka et al., 2019; Vos et al., 2011). As neither 4-4A1 nor 4–7B1,2 could ferment rhamnose, dulcitol, or raffinose, they were most likely to be L. paracasei subsp. paracasei. The fermentation pattern suggested that two isolates (80-5B3 and 80-5A), were L. rhamnosus as they could ferment rhamnose and dulcitol (Grześkowiak et al., 2011). To identity these isolates that cannot be discriminated from their similar phenotypes using an orthogonal approach, the 16S rRNA sequencing and MLST of the isolates were used.

The API ID 32 C test results of 15 yeast isolates indicated that they all belonged to the species *Saccharomyces cerevisiae* with 99.9% identity. To confirm the identify of yeast isolates, five representative yeast isolates were further analysed using the 26S rRNA sequencing.

3.3. 16S rRNA sequencing and MLST of LAB isolates

To identify the LAB species using 16S sequencing, the 16S regions from each isolate were amplified using PCR followed by Sanger sequencing. Comparison of these sequences with the GenBank database via BLAST showed that all four LAB isolates belonged to *Lactobacillus paracasei* subsp. *paracasei* species, each having 99.9% identity. MLST was used to obtain a higher resolution for taxonomic placement of the isolates. Phylogenetic analysis of the LAB isolates with 37 other
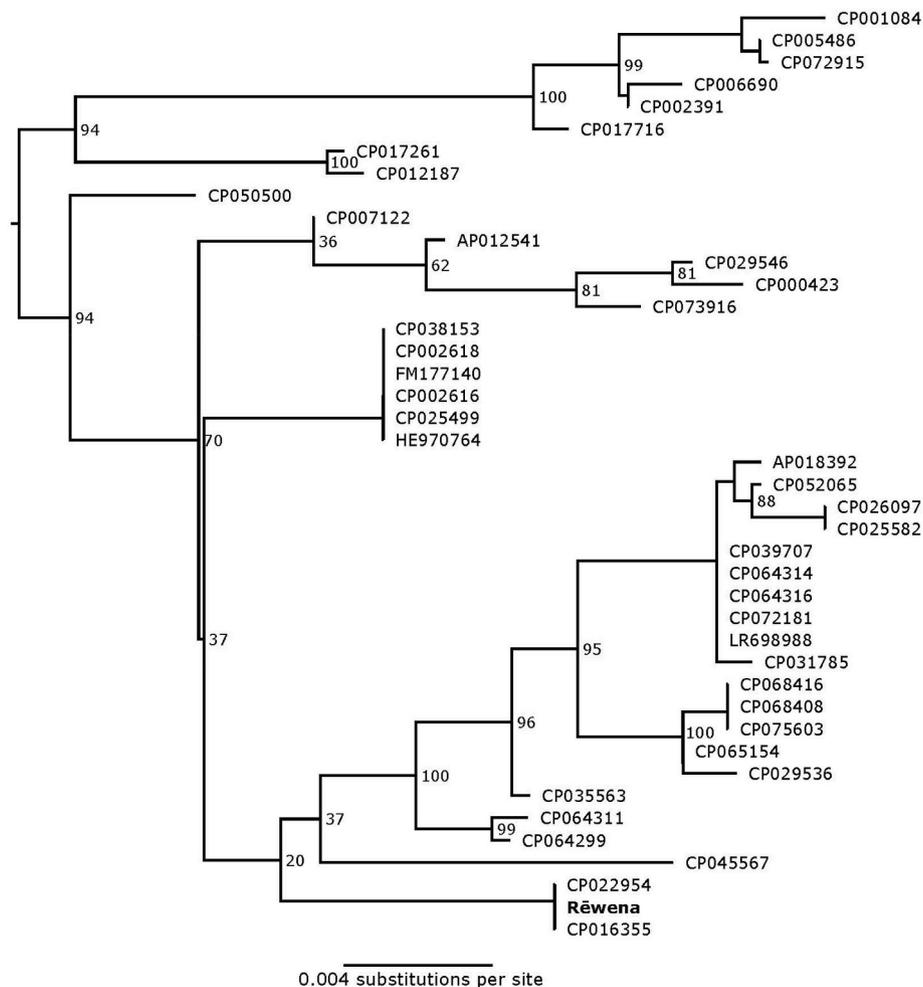
Fig. 1. Maximum likelihood tree based on the concatenated MLST sequences of Lactobacillus paracasei isolates.
Note: The Rēwena isolate is highlighted in bold. The scale bar shows the number of substitutions per base pair across a 4262 base pair alignment. The tree is midpoint rooted, and bootstrap values are shown at each node.

Lactobacillus casei strains indicated that the MLST genotypes of the LAB isolates were identical to those of *L. paracasei* subsp. *paracasei* IBB 3423 (NCBI CP022954) and *L. paracasei* subsp. *paracasei* F19 (NCBI CP016355) (Fig. 1). However, these strains undergo frequent intergenic recombination, as indicated by the maximum likelihood tree structure in Fig. 1 (Cai et al., 2007; de Las Rivas, Marcobal and Munoz, 2006). This suggested that the strains may differ considerably at the whole-genome level.

API 50 CHL test results for LAB 80-5A3 and 80-5B3 isolates suggested that they belonged to *L. rhamnosus*. However, the 16S rRNA sequencing indicated that the two LAB isolates belonged to *Lactobacillus paracasei* subsp. *paracasei* species. Ribosomal RNA (rRNA) sequencing is reported as accurate for discriminating LAB at the species level due to their conservative regions that help to identify species (Khalid, 2011). It is noteworthy to mention that similar phenotypes of LAB do not always imply corresponding similar genotypes. Although phenotypic methods are applied to identify food associated LAB, such methods are limited by their low discriminatory ability (Temmerman et al., 2004). Previous studies (Corsetti et al., 2001; Muyanja et al., 2003) reported the limitations of taxonomic discrimination in the identification of LAB isolates using phenotypic methods. Accordingly, isolates 80-5A3 and 80-5B3 should be assigned to *Lactobacillus paracasei* subsp. *paracasei*. Previous studies indicated the presence of both *L. paracasei* species and *L. rhamnosus* species in sourdough, moreover, *L. paracasei* species have been more commonly isolated from sourdough than *L. rhamnosus* species (Foschino et al., 2004; Luangsakul, Keeratipibul, Jindamorakot and Tanasupawat, 2009a; Weckx et al., 2010). Furthermore, depending on the desired taxonomic level of LAB isolates, MLST genotyping identified the two most similar strains to the tested LAB isolates as *L. paracasei* subsp. *paracasei* IBB 3423 and *L. paracasei* subsp. *paracasei* F19, both of which have identical MLST genotypes. 16S rRNA sequencing and MLST are considered as two complementary methods for taxonomic discrimination (Sacchi et al., 2005). The concordance of the genotypes of the isolates that were identified by MLST and 16S sequencing with the previously characterised strains suggested that the MLST genotypes were accurate, and furthermore, that MLST provided a more precise means of assigning taxonomy than phenotypic assays. However, the fact that these two strains were identical at the MLST loci does not preclude them from being considerably different at other genomic loci, many of which may affect phenotypes. Thus, although from a genetic standpoint both isolates were assigned to *L. paracasei* subsp. *paracasei,* further phenotypic testing may differentiate them from other *paracasei* strains.

Sourdough is an ecosystem that is dominated by LAB and yeast (De Vuyst, Harth, Van Kerrebroeck and Leroy, 2016; Zhang et al., 2015) which varies in sourdough from different regions due to factors such as the specific ingredients used, endogenous enzyme activities, process parameters, population divergence during back-slopping, and interactions between LAB and yeast (Weckx et al., 2010). *L. casei* and *L. paracasei* species have been frequently isolated from sourdough (Luangsakul, Keeratipibul, Jindamorakot and Tanasupawat, 2009b), however the two species have also been reported in dairy-related products (Desai et al., 2006). The microorganisms in sourdough have

been reported to have a lower diversity when back-slopping is used, resulting in mature sourdoughs being dominated by one type of LAB species (Lattanzi et al., 2013; Minervini et al., 2014). The potato starter culture used in the current study, is believed to have been propagated by back-slopping for nearly a century (T. Poi-poi Davy, personal communication, 26 April 2019). Therefore, the results from the study show that the potato starter culture was indeed dominated by a single LAB species, in this case *L. paracasei* subsp. *paracasei*.

3.4. The 26S rRNA sequencing of isolated yeast

26S rRNA sequencing of the yeast isolates identified all five as *Saccharomyces cerevisiae*, having 100% identity to *S. cerevisiae* strain Y169 (CP033481.1), confirming the results of the API ID 32 C test. *S. cerevisiae* has been widely identified in sourdough by 26S rRNA sequencing (Lattanzi et al., 2013; Zhang et al., 2011), and is also one of the most isolated species from back-sloping fermented sourdough (De Vuyst et al., 2016). Therefore, it is not surprising that the yeast in the potato starter culture belonged to the species *S. cerevisiae*.

4. Conclusion

LAB isolates obtained from the potato starter culture that is used to ferment Parāroa Rēwena sourdough bread belonged to the *L. paracasei* subsp. *paracasei* species, with the MLST data most closely matching *Lactobacillus paracasei* subsp. *paracasei* IBB 3423 and *L. paracasei* subsp. *paracasei* F19 (NCBI numbers: CP022954 and CP 016355, respectively). All the isolated yeast belonged to the species *S. cerevisiae* with the most probable strain being strain Y169.

CRediT authorship contribution statement

Jia Sun: Data curation, Conceptualization, Methodology, Software, Writing – original draft, Writing – review & editing. **Olin Silander:** Funding acquisition, Writing – review & editing, Data curation, Resources, Software, Methodology. **Kay Rutherfurd-Markwick:** Writing – review & editing, Conceptualization, Writing – review & editing, Funding acquisition. **Daying Wen:** Data curation, Software, Methodology. **Tanya Poi-poi Davy:** Resources, Conceptualization, Funding acquisition. **Anthony N. Mutukumira:** Writing – review & editing, Data curation, Resources, Conceptualization, Methodology, Project administration, Funding acquisition, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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