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Functional Properties of Microorganisms Isolated from Formulated Sourdough, Coconut Water Kefir, and Kefir

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Abstract: Recently, there has been a renewed interest in the fermentation of kefir grains using fruit-based substrates, such as coconut water. Kefir grains contain a mixture of lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeast, which have important probiotic capacity and play a vital role in improving the nutritional and functional properties of the new product being developed. The principal objective of this study was to determine the functional properties of the microorganisms identified and characterized from kefir, CWK, and sourdough fermented with coconut water kefir (CWKS), such as *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, *L. fusant*, *L. reuteri*, *L. kunkeei*, *Acetobacter aceti*, *A. lovaniensis*, *A. pasteurianus*, *Candida kefyr*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, *C. guilliermondii*, and *C. colliculosa*. In addition to identifying functional properties, such as glutamic acid production, phytase production, phytic acid degradation, and exopolysaccharide production, from this study, it was found that significantly high quantities of glutamic acid, exopolysaccharide, and phytase enzyme were detected in two LAB isolates, *Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum*.

Keywords: coconut-water-kefir-fermented sourdough; functional properties; glutamic acid; phytase; phytic acid; γ-aminobutyric acid; exopolysaccharide; bioactive

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1. Introduction

Sourdough fermentation can impart health-benefiting properties to bread beyond being a source of nutrients. Such functional properties of sourdough may include increased bioavailability of phytonutrients, decreased glycemic response, and an increase in dietary fibre [1,2]. Within the cereal matrix, microbial activities of LAB and yeasts can result in the production of nutritionally active compounds, such as peptides, amino acids (and amino acid derivatives, such as γ-amino butyric acid), and exopolysaccharides [3,4].

Sourdough fermentation leads to phytase-dependent dephosphorylation of phytic acid [5]. Phytic acid [myo-inositol hexakis (dihydrogenphosphate)] constitutes 1–4% by weight of cereal grains, being a source of myo-inositol and the major storage form of phosphorus. This molecule is highly charged, with six phosphate groups extending from the central myo-inositol ring.

However, phytic acid chelates several divalent nutritional minerals; hence, it is considered an antinutritional factor. Phytic acid also complexes the basic amino acid group of proteins, thus decreasing the dietary bioavailability of these nutrients [6,7]. Therefore, its degradation improves the availability of nutrients and minerals, such as phosphorous, magnesium, iron, calcium, and zinc. Some of the phytase activities are endogenous to cereals but activated by LAB acidification, whereas other phytase activities are directly produced by both yeasts and LAB [8]. In addition, the LAB in the sourdough produce bioactive compounds, volatile fatty acids, lactic acid, and amino acids during the fermentation process [9].

Glutamic acid is an amino acid that plays a very important role in taste perception, intermediary metabolism, and excitatory neurotransmission. It plays a pivotal role in the

gastrointestinal tract during gastric-phase digestion and enhances gastric exocrine secretion. It is also a precursor for γ-amino butyric acid (GABA), glutathione, arginine, and proline. GABA possesses several well-known physiological functions (i.e., anti-hypertension and anti-diabetic), and glutathione plays a key role in protecting the mucosa from peroxide damage and dietary toxins. A study by Zareian et al. (2013) [10] reported that *L. plantarum* has the potential to synthesize both glutamic acid and GABA in fermented foods. LAB also play a role in increasing the shelf-life and safety of foods, improving food texture, and contributing to the nutritional value of food products. The LAB can impart pleasant sensory properties to sourdough bread. Li and Cao et al. 2010 [11] have reported that production of L-glutamic acid and GABA (γ-aminobutyric acid) was of utmost importance to producing a functional food product. Komatsuzaki et al. (2005) [12] have reported that during fermentation, the production of glutamic acid was positively correlated with an increase in GABA concentration. Thus, using LAB capable of producing glutamic acid might facilitate production of functional foods rich in bioactive molecules, such as GABA [13].

The main metabolic activities of sourdough LAB that influence the nutritional importance of sourdough are the proteolytic activity [14], the formation of volatile compounds and antibacterial and anti-mold compounds [15], as well as exopolysaccharide (EPS)-producing characteristics [16]. Exopolysaccharides (EPS) are microbial polysaccharides produced outside of the cell wall [17]. They consist of dietary oligosaccharides, which are non-digestible and aid in modulating the activity and composition of the intestinal microflora. EPS may also contain a variety of proteins, glycoproteins, glycolipids, and, in some cases, surprising amounts of extracellular DNA (e-DNA) [18].

Sourdough LAB synthesize a variety of EPS through the activity of glycosyltransferases. A previous study carried out on EPS suggests that some of the polymers from the fermentation of cereal foods by LAB may be available for food applications and processing [19,20]. Synthesis of EPS (glucans and fructans) with prebiotic potential has been reported for sourdough fermentation of sorghum and wheat flours by LAB, such as *L. frumenti*, *L. pontis*, *L. acidophilus*, and *L. reuteri* [21–23]. Kralj et al. (2002) [24] reported that by adding 12% sucrose to the dough during fermentation, *L. reuteri* strain 121 can form two types of EPS, a 4,6-disubstituted ά-glucan (reuteran) and a levan. Glucansucrase and fructosyltransferase are the enzymes responsible for the synthesis of the abovementioned EPS [25].

EPS acts as a hydrocolloid to improve the overall textural properties of the sourdough bread produced. Cereal-based LAB species have been associated with the formation of homopolymeric EPS, but recent studies also showed the production of heteropolymeric EPS by sourdough isolates, which were shown to alter the physicochemical properties of sourdough [26]. Identification of the EPS-producing LAB in sourdough LAB could help improve the quality of sourdough.

Therefore, the objective of this study was to establish the functional properties of the LAB, yeast, and acetic acid bacteria isolated from the coconut-water-kefir-fermented sourdough, CWK, and kefir. This study specifically aims to determine the following properties of the isolates, such as glutamic acid production, phytase enzyme activity, and exopolysaccharide production.

2. Materials and Methods

2.1. LAB, AAB, and Yeast Species

All of the LAB, AAB, and yeast strains used in this study (Table 1) were isolated and identified from kefir, CWK, and CWK-fermented sourdough. Because they were stored at −80 °C with 20% glycerol, they were first revived by growing them in MRS broth (Difco, Auckland, New Zealand) and acetic acid bacteria broth (DIFCO, New Zealand) and incubated for 48 h at 30 °C (LabServe incubator, Auckland, New Zealand).

Table 1. Isolates used in the study.

Type	Origin	Isolates
LAB	Kefir, CWK, CWK-fermented sourdough	*Limosilactobacillus fermentum* *Lactiplantibacillus plantarum* *Lactobacillus fusant* *Lactobacillus reuteri* *Lactobacillus kunkeei*
AAB		*Acetobacter aceti* *Acetobacter lovaniensis* *Acetobacter pasteurianus*
Yeast		*Candida kefyr* *Rhodotorula mucilaginosa* *Saccharomyces cerevisiae* *Candida guilliermondii* *Candida colliculosa*

2.2. Cultures and Culture Media

The samples used for microorganism isolation were taken from fresh young coconut water (Countdown, Auckland, New Zealand) kefir grain powder purchased from Body Ecology™, Auckland, New Zealand, and CWK-fermented sourdough. Sourdough (600 g) was prepared using CWK (300 mL), table salt (3 g), and canola oil (1.5 mL). In total, 1 g of sourdough sample was taken every 24 h of fermentation, which was suspended in 9 mL of deionized water using a stomacher (or a mechanical mixer) [27]. All of the identified species of LAB, acetic acid bacteria, and yeasts (Section 3.1) included in this study (Table 1) were isolated and identified from kefir, CWK, and CWK-fermented sourdough as per the study by Limbad et al. (2024) [27]. The phytic-acid-containing medium was prepared from phosphate-free minimal medium, supplemented with 2 g/L of phytic acid dipotassium salt of the highest available purity (Sigma-Aldrich Inc., Auckland, New Zealand). The quantity of free phosphate in the phytic acid was established to be <5% (*w*/*w*) in all formulations used, in accordance with the manufacturer's instructions. All media were prepared as 10-fold-concentrated stock solutions and filter sterilized (Filtropure, 0.45 µm, Sarstedt, Germany) instead of autoclaving, which releases orthophosphate from phytate [28]. To obtain the corresponding solid medium, Bacteriological Agar without phosphorus (15 g/L) (Oxoid Ltd., Wilmington, DE, USA) was suspended in water (90% of the final volume) and autoclaved at 121 °C for 15 min, which was used for yeast isolates. After cooling to 45 °C, 10-fold-concentrated phosphate containing minimal medium, phosphate-free minimal medium, or phytic-acid-containing medium was added. All media were formulated with deionized water.

All of the yeast isolates were grown on 10 mL of phosphate containing minimal medium and incubated at 25 °C for 48 h in a LabServ incubator (Thermo Fisher, Auckland, New Zealand). Yeast cells were harvested through centrifugation (DuPont Sorvall instruments, RC5C, Wilmington, DE, USA) at 4000× *g* for 5 min. The pellet obtained was washed three times with 10 mL of sterile sodium chloride solution (9 g/L). The cell suspensions were standardized at an optical density $(O.D.)_{540nm}$ reading of 2.0 using a spectrophotometer (Pharmacia Biotech Ultrospec 2000, Thermo Fisher, Auckland, New Zealand) [29].

2.3. Determination of Phytase Production by LAB, AAB, and Yeast Isolates

2.3.1. Phytase Assay: Agar Assay for LAB and AAB

The pure cultures of LAB and AAB strains were grown, respectively, on MRS broth and acetic acid bacteria broth (Difco, New Zealand) and then incubated for 48 h at 35 °C. The phytase studies were carried out on modified MRS broth (MRS-MOPS) in which the inorganic phosphate (potassium dihydrogen phosphate) was replaced by 0.65 g/L of sodium phytate and 0.1 M 3-[N-Morpholino] propane sulfonic acid (MOPS, Thermo Fisher,

New Zealand). In the MRS broth, the contents of beef extract, glucose, and yeast extract were reduced to 4 g/L, 10 g/L, and 2 g/L, respectively, to reduce the final phosphate concentration and promote enzyme synthesis [30]. Additionally, MRS-MOPS broth with added arginine and glycine (10 g/L) and supplemented with lactose (10 g/L) instead of glucose (MRS-MOPS + Amino acid) was inoculated with each active culture [31]. MRS-MOPS and MRS-MOPS + Amino acid agar plates were prepared using the same broth recipe but with the addition of agar at 1%.

No calcium carbonate was present in MRS-MOPS or MRS-MOPS + Amico acid agar media, but they were all supplemented with 1% of hexacalcium phytate (Sigma-Aldrich, New Zealand).

The MRS-MOPS and MRS-MOPS + Amino acid agar media were thereafter inoculated with 5% v/v overnight cultures of LAB and AAB strains (with absorbance of 2 O.D recorded at 600 nm wavelength). The inoculum was spread across a uniform area, in a circular manner, over a diameter of 5 cm. The plates were incubated for 48 h at 30 °C. After incubation, the colonies were washed from the agar surface using double-distilled water, and Petri plates were flooded with 2% (w/v) aqueous cobalt chloride solution [32]. This was allowed to stand for 5 min at room temperature, after which the cobalt chloride solution was replaced with a newly formulated solution containing equal volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium metavanadate solution. After 5 min of incubation, the ammonium molybdate/ammonium vanadate solution was separated, and the plates were studied for the zone of phytate hydrolysis.

2.3.2. Phytase Assay: Enzyme Activity Assay for LAB and Acetic Acid Bacteria

All of the strains of LAB and AAB that exhibited some ability to hydrolyze phytase in the solid medium were chosen to perform the phytase enzyme activity using liquid medium. Three different liquid media, namely, Chalmers broth, Chalmers broth supplemented with 1% of sodium phytate, and Chalmers broth supplemented with 2% of calcium chloride, were used for this assay (Sigma-Aldrich, New Zealand). Each isolate of LAB and AAB was inoculated into triplicate bottles of each broth.

After 24 h of incubation at 30 °C, all of the broths were centrifuged at 10,000× g for 10 min at room temperature, and 1 mL of supernatant was collected. This was mixed with 600 µL of a reaction substrate (3 mM sodium phytate in 0.2 M sodium acetate buffer, pH 4.0) and incubated at two different temperatures of 30 °C and 44 °C for 45 min and 75 min, respectively. The reaction was stopped by adding 750 µL of a 5% weight/volume trichloroacetic acid (Sigma T6399) solution. The released inorganic phosphate was evaluated by measuring the absorbance at 700 nm after the addition of 750 µL of fresh color reagent. The color reagent consisted of four volumes of 1.5% w/v ammonium molybdate (Sigma A7302) in a 5.5% v/v sulphuric acid solution and one volume of a 2.7% w/v ferrous sulphate (Sigma F7002) solution.

Phytase activity was measured in terms of inorganic orthophosphate released from phytate (µmol for min). One unit of phytase activity (U/mL) was defined as the amount of enzyme required to liberate 1 µmol of inorganic phosphate per minute under assay conditions [33].

All of the results were compared to a standard curve prepared with inorganic phosphate-di-potassium hydrogen phosphate (K_2HPO_4).

2.4. Determination of Phytase Activity for Yeast Isolates

2.4.1. Extraction of Phytase Enzyme

All of the yeast isolates were grown in 10 mL of phosphate-containing minimal medium incubated at 25 °C for 48 h in a LabServ incubator (Thermo Fisher, New Zealand). All of the yeast cells were harvested through centrifugation (DuPont Sorvall instruments, RC5C) at 4000× g for 5 min. The pellet obtained was washed three times with 10 mL of sterile sodium chloride solution (9 g/L). The absorbance at 600 nm was determined using a spectrophotometer (Pharmacia Biotech Ultrospec 2000 Spectrophotometer) [33].

Extracellular phytase activity (activity of the enzymes that are not bound to the yeast cells but are present in the medium) was measured by collecting the supernatant after centrifugation of the yeast cells grown in the media and filter sterilizing (0.2 µm pore size, Filterpure, Auckland, New Zealand) to remove any cell debris present in the medium.

Intracellular phytase enzyme (present inside the yeast cell wall and cytoplasm) was extracted through a slight modification to a method described by Ciriacy and Breitenbach (1979) [34].

The yeast cell pellet was suspended again in 20 mL of crude extract buffer kept at a low temperature (50 mM imidazole, 0.5 mM dithiothreitol, pH 7), followed by two washes with 20 mL of the same buffer. These suspensions were then moved to Eppendorf tubes and centrifuged in an Eppendorf centrifuge 5810 R at 10,000× *g*, 4 °C for 20 min to remove any remaining cell debris. After centrifugation, the supernatants were filtered using a filter with a pore size of 0.2 µm and concentrated to 250 µL in an Amicon Ultra centrifugal device (Sigma-Aldrich, Auckland, New Zelanad), then placed on ice. This process was repeated three times for each yeast strain tested to ensure accuracy.

2.4.2. Enzyme Assay

A volume of 10 µL from the sample was combined with 40 µL of 0.1 M sodium acetate buffer (composed of 0.2 M acetic acid and 0.2 M sodium acetate, pH 5.0), supplemented with phytic acid dipotassium salt to achieve a final concentration of 2 g/L phytic acid. Negative control samples were prepared by mixing with sodium acetate buffer lacking phytic acid. Samples were incubated at 30 °C, and the reactions were halted immediately (at time, T = 0 min) and after 5, 10, 15, 30, and 60 min by adding 50 µL of 10% trichloracetic acid. The liberated phosphate was determined through a method described by Heinonen and Lahti (1981) [35] by adding 800 µL of acid molybdate reagent (1 volume of 10 mM ammonium heptamolybdate–tetrahydrate, 1 volume of 2.5 M sulphuric acid, and 2 volumes of acetone).

The absorbance reading at 335 nm was measured by using sodium acetate buffer (without phytic acid) and trichloroacetic acid as the blank. A standard curve of phosphate (range of 0 µmol/mL to 3 µmol/mL) was prepared with sodium acetate buffer and measured in the same conditions as the enzyme samples [36,37].

2.4.3. Calculation of Enzyme Activity for Yeast Cells

A single unit of phytase activity refers to the quantity of protein capable of liberating 1 µmol of inorganic phosphate within a minute. Phytase activities were assessed in terms of both specific activity (Unit/mg of protein) and volumetric activity (Unit/mL of yeast culture). All experiments were conducted three times, and the average results are reported. The activities were calculated according to Bergmeyer (2012) [38]:

$$\text{Specific activity (Unit/mg protein)} = V \times \Delta cPO_4 \times v^{-1} \times cprot^{-1} \times \Delta t^{-1}$$

$$\text{Volumetric activity (Unit/mL of culture liquid)} = V \times \Delta cPO_4 \times v^{-1} \times \Delta t^{-1} \times (100\ mL)^{-1}$$

where "V" is the total sample volume measured in mL; "ΔcPO_4" is the phosphate concentration that has changed with the time interval, measured in µmol; "v" is the volume of the crude extract measured in mL; "cprot" is the protein concentration measures in mg/mL; and "Δt" is the time interval measured in min.

2.5. Determination of Glutamic Acid Concentration Produced by the Individual Strains of LAB, Yeast, and Acetic Acid Bacteria

All of the identified strains of LAB, AAB, and yeasts were grown in MRS broth, acetic acid bacteria broth, and Malt extract broth, respectively. The LAB and AAB were incubated at 30 °C, and yeast strains were incubated at 25 °C in a LabServ incubator, New Zealand, for 48 h. All of the cultures were centrifuged at 5000× *g* for 10 min to collect the cell-free supernatant. This was sampled in triplicate to carry out the LC-MS chromatographic analysis for determining the glutamic acid concentration produced by individual strains/isolates.

A 1.4 mL volume of 50% methanol containing 10 mg L-1 of 2,3,3,3-d4-alanine [d4A] was added to the samples and mixed in a microcentrifuge tube.

The tubes were vigorously mixed to achieve a uniform blend. Subsequently, they underwent centrifugation at 10,000× *g* for 5 min at 4 °C using the Z216MK centrifuge from HERMLE Labortechnik GmbH, Wehingen, Germany. A 10 µL portion of the extract was subjected to pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, following a technique adapted from Salazar et al. (2012) [39]. The LC-MS setup comprised components from the Agilent 1260 Series (Santa Clara, CA, USA), including a G1311B quaternary pump, a G1329B thermostatted autosampler, and a G1330B thermostatted column compartment (Agilent Technologies, Santa Clara, CA, USA). Mobile phase A consisted of 0.6% formic acid in ultrapure water, while mobile phase B comprised 0.1% formic acid in acetonitrile. The column utilized was a Phenomenex Kinetex EVO C18 column (Thermo Fisher, Auckland, New Zealand), measuring 2.1 × 150 mm with 1.7 µm diameter packing material, maintained at 25 °C. The chromatographic gradient was held at 1.5% B for 1 min, then increased to 13% B at eight minutes, 17% B at 15 min, and 80% B at 16 min before reverting to 1.5% B at 17.5 min. The flow rate was set at 300 µL min^{-1}, the total run time was 28 min, and the injection volume was 5 µL. Detection was performed using an Agilent 6420 triple quadrupole mass spectrometer equipped with an Agilent Multimode Ionization source operating in positive electrospray mode. Optimal Multiple Reaction Monitoring [MRM] transitions were established using Agilent MassHunter Optimizer B06.00 software. Derivatized amino acid peak areas were normalized to the recovery of d4A and quantified by referencing a dilution series of external standards prepared from a commercial glutamic acid standard (Sigma product A9906, Sigma-Aldrich Pty. Ltd., Sydney, Australia). Data were collected and processed using Agilent MassHunter software.

2.6. Determination of Exopolysaccharide Production in LAB and Acetic Acid Bacteria Strains

2.6.1. Screening for Exopolysaccharide (EPS)-Producing Microorganisms

Verification of EPS production by the strains was performed by observing growth on modified Chalmers agar [40]. Modified Chalmers agar was prepared through the addition of sucrose (5% weight/volume). No calcium carbonate was included in the agar when made. All of the carbohydrates were filter-sterilized before they were added to the medium by using filters with a 0.45 µm pore size (Swinney syringe filters, Auckland, New Zealand).

Production of EPS was detected by the presence of slimy colonies on the agar plates after incubation at 30 °C for 72 h (LabServe incubator, New Zealand). The colonies were examined by touching them with a sterile toothpick to observe the ropy EPS phenotype that produces long visible strings [41,42]. The positive strains detected using the primary assay were also tested on modified Chalmers agar without the addition of calcium carbonate and supplemented with 5% sucrose. The strains were incubated at 30 °C for 3 days, and the ropy colonies were evaluated again, as described above. All of the tests were performed in triplicate for each strain of LAB and AAB.

2.6.2. Quantification of EPS Production

All of the strains of LAB, AAB, and yeasts that tested positive for EPS production in a solid substrate were selected for quantitative analysis for EPS production. All of the strains were grown in their respective broths (MRS broth for growing LAB and acetic acid bacteria broth for growing AAB, manufactured by Difco, New Zealand) for 48 h at 30 °C in a LabServe incubator (New Zealand).

All of the inocula were standardized by using a spectrophotomer, on which the absorbance reading was taken and adjusted to 2 O.D. through dilution or concentration of all of the samples (in triplicate). A Pharmacia Biotech Ultrospec 2000 Spectrophotometer UV/Visible (Thermofisher, Auckland, New Zealand) light Reader was used at the wavelength of 600 nm. One milliliter of the culture acquired from each of the isolates had approximately 5 × 10^7 LAB and AAB isolates. This was determined using a spread plate technique. Therefore, 1 mL was taken from each strain in triplicate and streaked onto a

Chalmers agar with 5% sucrose with and without the addition of 0.5% *w*/*v* of yeast extract. This was done to ascertain the effect of yeast extract on EPS production. The plates were incubated at 30 °C for 48 h. After incubation, all of the colonies that produced EPS were washed repeatedly at 5000 g for 5 min at room temperature (DuPont Sorvall instruments RC5C, Fiberlite F21–8x50y rotor, Thermofisher, Waltham, MA, USA) with 30 mL of deionized water until the visible ropiness disappeared. The ropy colonies were collected in sterile 100 mL falcon tubes.

The falcon tubes were centrifuged at 5000× *g* for 10 min at room temperature to collect the cell pellet (DuPont Sorvall instruments RC5C, Fiberlite F21–8x50y rotor, Thermofisher USA). This was followed by the addition of two volumes of 98% (*v*/*v*) ethanol to precipitate the EPS. This was left to stand overnight at 4° C, followed by centrifugation at 5000× *g* for 10 min at 4 °C temperature. The pellet was retained and re-suspended in 1 mL of double-distilled water. The suspension was freeze-dried on a benchtop freeze drier (Virtis Advantage Pro from SP Scientific, Warminster, PA, USA) for 24 h and weighed to quantify the EPS using an analytical balance (Thermo Fisher, New Zealand).

The amount of EPS obtained was expressed as polymer dry mass in mg/kg of the wet medium [43,44].

2.7. Statistical Analysis

At least five individual replicates of each of the experimental sets were prepared for statistical analysis. All of the results are expressed as means of values ± standard deviation of three separate determinations. One-way and two-way analysis of variance (ANOVA) were applied to determine the level of significance. When the ANOVA was significant, the means were separated through pairwise comparison using Tukey's (HSD) test or Fisher's (LSD) test at a 5% significance level. All statistical analyses were performed using the Statistical Analysis System—XLSTAT-MX version 2012.4.02 (Addinsoft, New York, NY, USA).

3. Results and Discussion

3.1. Determination of Glutamic Acid Concentration for LAB, AAB, and Yeast Isolates Using the LCMS Analysis Method

To investigate the concentration of glutamic acid produced by each isolate of LAB, ABB, and yeast, LCMS analysis was carried out. From Table 2, it can be observed that a significantly high concentration of glutamic acid was produced by *Lactiplantibacillus plantarum* (264.89 ± 8.57 µMoles/L), followed by *Limosilactobacillus fermentum* (260.38 ± 12.09 µMoles/L) and *Lactobacillus reuteri* (251.16 ± 10.61 µMoles/L) $p < 0.05$. Gobbetti et al. (1994) [45] have reported that using *Lactiplantibacillus plantarum* in sourdough fermentation caused a considerable increase in the total concentration of free amino acids. Zareian et al. (2012) [13] reported *Lactiplantibacillus plantarum* as a glutamic acid producer. A similar study by Pozo-Bayón et al. (2005) [46] detected the presence of glutamic acid by *Lactiplantibacillus plantarum* strains J-39 (19.17 mg/L) and J-51 (16.82 mg/L) in wine after malolactic fermentation by LAB. A study carried out by Coda, Rizzello, and Gobbetti (2010) [47] reported considerably high glutamic acid concentrations of 107 ± 20 mg/L in sourdoughs made with common wheat flour and fermented with *Lactiplantibacillus plantarum* strain C48.

Table 2. Glutamic acid concentration determined using LCMS for each isolate in µMoles/L. (a–g determine significant differences within a column).

Isolates	Glutamic Acid Concentration (µMoles/L)
Limosilactobacillus fermentum	260.38 ± 12.1 a,b
Lactiplantibacillus plantarum	264.89 ± 8.5 a,b
Lactobacillus fusant	228.96 ± 12.7d
Lactobacillus reuteri	251.16 ± 10.6 b,c
Lactobacillus kunkeei	239.08 ± 10.8 c,d
Acetobacter aceti	155.28 ± 4.8 e
Acetobacter lovaniensis	164.37 ± 4.1 e

Table 2. *Cont.*

Isolates	Glutamic Acid Concentration (μMoles/L)
Acetobacter pasteurianus	159.52 ± 0.7 e
Candida kefyr	120.83 ± 0.9 f,g
Rhodotorula mucilaginosa	133.35 ± 0.8 f,g
Saccharomyces cerevisiae	129.37 ± 3.8 f,g
Candida guilliermondii	114.29 ± 1.9 g
Candida colliculosa	128.18 ± 7.1 f,g

AAB strains (*Acetobacter aceti*, *Acetobacter lovaniensis*, and *Acetobacter pasteurianus*) produced average quantities between 155.28 ± 4.84 μMoles/L and 164.37 ± 4.11 μMoles/L of glutamic acid (as shown in Table 2). Significantly low concentrations of glutamic acid are produced by all of the individual yeast species, with *Candida guilliermondii* significantly producing the lowest (114.29 ± 1.95, $p < 0.05$).

Gobbetti et al. (1994) [48] reported the production of enhanced amounts of glutamic acid by *S. cerevisiae* strain 141 during sourdough fermentation.

3.2. Phytate Zone of Hydrolysis

All LAB, AAB, and yeast strains isolated from kefir, CWK, and CWK-fermented sourdough were screened for their phytate-degrading ability using two different types of media: modified MRS agar (MRS-MOPS) in which inorganic phosphate was replaced by sodium phytate (0.65 g/L, Sigma) and 0.2% $CaCl_2$, and MRS-MOPS + AA supplemented with 10 g/L of arginine and glycine.

All of the isolates were incubated for 48 h at 30 °C. As shown in Table 3, all were positive for phytate hydrolysis by exhibiting a zone of clearance (translucent) around the inoculated area on the agar. A counterstaining method was used by flooding the agar with aqueous cobalt chloride, which helps determine the false positives for phytate-negative microorganisms. The exact mechanism of the counterstaining procedure is not yet known. However, it is estimated that phytate can form complexes with cobalt and metals and that it has a pH dependence on these complexes, with relative binding of different cations with phytate [49].

Table 3. Phytate zone of hydrolysis by LAB, AAB, and yeast on MRS-MOPS and MRS-MOPS supplemented with amino acids grown for 48 h at 30 °C. Different letters in each column indicate statistical differences between strains ($p < 0.05$). The data are expressed as the mean and standard deviation for the two independent experiments.

LAB, AAB, and Yeast Isolates	Zone of Hydrolysis (Diameter of the Zone of Hydrolysis in mm)	
	Average MRS-MOPS	Average MRS-MOPS + Amino Acids
Limosilactobacillus fermentum	14.2 ± 0.1 a	12.7 ± 0.1 a
Lactiplantibacillus plantarum	13.8 ± 0.1 a	12.4 ± 0.1 b
Lactobacillus fusant	12.1 ± 0.5 a	11.9 ± 0.1 d
Lactobacillus reuteri	13.6 ± 0.1 a	12.1 ± 0.1 c,d
Lactobacillus kunkeei	13.7 ± 0.1 a	12.2 ± 0.1 b,c
Acetobacter aceti	8.5 ± 0.2 c	8.4 ± 0.1 f
Acetobacter lovaniensis	9.3 ± 0.1 b	8.9 ± 0.2 e
Acetobacter pasteurianus	8.8 ± 0.2 b,c	8.3 ± 0.1 f
Candida kefyr	3.0 ± 0.1 d	2.5 ± 0.2 g
Rhodotorula mucilaginosa	2.7 ± 0.1 d	2.4 ± 0.3 g
Saccharomyces cerevisiae	2.73± 0.2 d	2.5 ± 0.1 g
Candida guilliermondii	2.4 ± 0.2 d	2.5 ± 0.1 g
Candida colliculosa	2.3 ± 0.1 d	2.4 ± 0.2 g

After counterstaining, all of the phytase-positive LAB, AAB, and yeast displayed a halo ranging between 2.3 mm and 14.24 mm in diameter. *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, *Lactobacillus fusant*, *Lactobacillus reuteri*, and *Lactobacillus kunkeei*

had significantly larger zones of hydrolysis that ranged between 14.24 ± 0.12 mm and 13.73 ± 0.08 mm ($p < 0.05$). LAB, AAB, and yeast were tested for their ability to degrade sodium phytate in the presence of calcium chloride. The supplemented calcium aids in the phytase enzyme activity and does not itself take part in the reaction [49]. The phytate degradation ability of all of these isolates can be attributed to the presence of the phytase enzyme, which degrades the available phytate (sodium phytate) in the agar in the presence of calcium chloride, because no white precipitate was observed around the zone of enzyme-specific phytate hydrolysis [50].

Acetobacter species had a significantly smaller hydrolysis zone compared to those of LAB, with values between 8.5 ± 0.2 mm and 9.37 ± 0.15 mm ($p < 0.05$). No previous study has reported phytate hydrolysis by AAB according to the previous literature. The ability to utilize phytate can thus be attributed to the production of the phytase enzyme by AAB.

A significantly smaller zone of hydrolysis was obtained for all yeast species, which ranged between 2.3 ± 0.17 mm and 3.07 ± 0.15 mm ($p < 0.05$). Positive yeast strains for phytate hydrolysis have been previously reported by Howson and Davis (1983) for *S. cerevisiae*, a GRAS (Generally Recognized as Safe) organism used in food for human consumption [51]. Degradation of phytate by phytase produced by *S. cerevisiae* is valuable in the industrial production of food from plant seed meals [52]. Tsang (2011) [53] showed that two out of the three isolates of *C. kefyr* and three out of four isolates of *C. guilliermondii* tested positive for phytate hydrolysis. Additionally, Caputo, Visconti, and De Angelis (2015) [54] reported that *C. colliculosa* possessed phytate hydrolysis ability. Phytase activity has also been reported to be positive for *Rhodotorula mucilaginosa* [55].

3.3. Phytase Enzyme Activity Assay

Each of the LAB, AAB, and yeast isolates that had tested positive for phytate hydrolysis (Table 3) were subjected to phytate assay in liquid medium. In this assay, sodium phytate in the presence of calcium was used to modulate the enzymatic activities of phytate-degrading enzymes in microorganisms [56,57]. One percent of sodium phytate added to each medium of growth tested did not act as an inhibitor of phytate-degrading enzymes. In fact, the phytase activity of all of the phytate-degrading strains was detected in all three media used, as reported in Table 4. Among LAB, AAB, and yeast, the highest phytase values were found for *Limosilactobacillus fermentum* (4052.53 ± 171.14 U/mL; $p \leq 0.05$) and *L. plantarum* (3151.83 ± 383.07 U/min; $p \leq 0.05$) grown on Chalmers broth and Chalmers broth supplemented with either 1% sodium phytate or 2% calcium chloride (Table 4).

Table 4. Phytase enzyme activity of LAB, AAB, and yeast on Chalmers broth and Chalmers broth supplemented with either 1% sodium phytate or 2% calcium chloride. Letters a–k represent significant differences among each isolate within the column.

Isolates	Average Phytase Activity (U/mL) in Chalmers Broth	Average Phytase Activity (U/mL) in Chalmers Broth + 1% Sodium Phytate	Average Phytase Activity (U/mL) in Chalmers Broth + 2% $CaCl_2$	Overall Average Phytase Activity (U/mL)
Limosilactobacillus fermentum	4052.2 a	4223.8 a	3881.5 a	4052.5 ± 171.1 a
Lactiplantibacillus plantarum	2717.9 b	3443.3 b	3294.1 b	3151.8 ± 383.0 b
Lactobacillus fusant	2497.3 d	2948.0 c	2495.2 e	2646.8 ± 260.8 c
Lactobacillus reuteri	2628.5 c	2875.3 d	2510.7 d	2671.5 ± 186.0 c
Lactobacillus kunkeei	2456.5 e	2437.1 e	2678.4 c	2524.0 ± 134.0 c,d
Acetobacter aceti	2373.9 f	2389.4 g	2403.6 f	2389.0 ± 14.8 d
Acetobacter lovaniensis	2375.2 f	2380.4 h	2395.9 g	2383.8 ± 10.7 d
Acetobacter pasteurianus	2376.5 f	2394.6 f	2392.0 g	2387.7 ± 9.7 d
Candida kefyr	2356.8 g	2361.0 i,j	2366.2 j	2361.3 ± 4.7 d
Rhodotorula mucilaginosa	2355.9 g	2357.2 k	2367.5 i,j	2360.2 ± 6.3 d
Saccharomyces cerevisiae	2354.6 g	2358.4 j,k	2371.3 h,i	2361.5 ± 8.7 d
Candida guilliermondii	2356.8 g	2359.7 j,k	2372.6 h	2363.9 ± 8.4 d
Candida colliculosa	2358.4 g	2363.6 i	2370.1 h,i,j	2364.8 ± 5.8 d

It was reported previously that all tested strains of LAB exhibited the production of phytase between 2.6 and 146 U/mL in terms of inorganic orthophosphate released [58].

Positive phytase production between 0.53 and 0.74 U/mL has been reported for *L. plantarum* T211, *L. plantarum* H10, and *L. plantarum* H5, and *L. plantarum* L3 has been previously reported by Anastasio et al. (2010) [59] for its growth on all three types of Chalmers broths (Chalmers broth and Chalmers broth supplemented with either 1% sodium phytate or 2% calcium chloride). Phytase production of 110.6 U/mL by *Limosilactobacillus fermentum* DC400 incubated for 24 h at 37 °C has been reported by De Angelis et al. (2003) [50], along with its capability to hydrolyze the available phytate in the medium. The phytase activity in this study is higher when compared to the literature, which could be due to the longer incubation time of 48 h in contrast to 24 h used by De Angelis et al. (2003) [50]. Yildirim and Arici (2019) [60] have reported phytase activity for *Lactiplantibacillus plantarum* ELB78 of 797.88 U/mL. Sumengen, Dincer, and Kaya (2013) [61] reported extracellular phytase activity of 984.50 U/mL from *Lactiplantibacillus plantarum* isolated from a fermented product known as "shalgam". This is significantly lower than the value obtained in this study for *L. plantarum*, which could be due to the longer fermentation time of the sourdough sets.

When compared with LAB, AAB strains showed significantly lower phytase activity ranging between 2389.02 ± 14.84 U/mL for *A. aceti* and 2383.86 ± 10.74 U/mL for *A. lovaniensis* ($p \leq 0.05$), followed by yeast strains with phytase activity ranging between 2360.21 ± 6.36 U/mL for *R. mucilaginosa* and 2364.08 ± 5.82 U/mL for *C. colliculosa* ($p \leq 0.05$).

Phytase activity has been reported for *S. cerevisiae* by Turk et al. (2000) [62] and for various other Candida species, including *C. kefyr*, *C. guilliermondii*, and *C. colliculosa*. Maximum phytase activity reached 205.4 U/mL for *Rhodotorula mucilaginosa* as reported by Yu, Wang, and Liu (2015) [55].

3.4. Screening for EPS Production

A method reported by Ruas-Madiedo et al. (2008) and Vescovo et al. (1989) for detecting EPS production was used [41,42]. EPS was detected through the examination of slimy colonies on the plate until 72 h of incubation at 30 °C on the abovementioned media. The positive colonies were further confirmed phenotypically through the formation of strings upon touching the colonies with a toothpick aseptically.

LAB, AAB, and yeast strains were considered positive for EPS production if they formed slimy colonies on Chalmers agar (CA) in the presence of 5% sucrose and without $CaCO_3$ (CA + S), CA supplemented with 5% sucrose and no yeast extract (YE), and CA supplemented with 5% sucrose and 0.5% YE (Table 5).

Table 5. This table reports only the species that showed EPS production in CA + S, CA + S − YE, and/or CA + S + YE.

Isolates	Qualitative EPS			
	Chalmers Agar (CA) + $CaCO_3$	**Chalmers Agar (CA) + 5% Sucrose without $CaCO_3$**	**Chalmers Agar + 5% Sucrose and No Yeast Extract**	**Chalmers Agar + 5% Sucrose + 0.5% wt/vol Yeast Extract**
Limosilactobacillus fermentum	-	++	++	++
Lactiplantibacillus plantarum	-	++	++	++
Lactobacillus fusant	-	-	-	-
Lactobacillus reuteri	-	++	++	++
Lactobacillus kunkeei	-	+	+	+
Acetobacter aceti	-	+	+	+
Acetobacter lovaniensis	-	-	-	-
Acetobacter pasteurianus	-	+	+	+
Candida kefyr	-	-	-	-
Rhodotorula mucilaginosa	-	+	+	+
Saccharomyces cerevisiae	-	-	-	-
Candida guilliermondii	-	+	+	+
Candida colliculosa	-	-	-	-

CA + S: modified Chalmers agar without $CaCO_3$ and with sucrose (5% wt/vol) as the carbon source. CA + S − YE: modified Chalmers agar without $CaCO_3$ and with sucrose (5% wt/vol) as the carbon source without addition of YE. CA + S + YE: modified Chalmers agar without $CaCO_3$ and with sucrose (5% wt/vol) as the carbon source with 0.5% wt/vol YE. LAB and AAB: incubated at 30 °C with 5% CO_2 and yeast incubated at 28 °C. "++": EPS (slime) production after 24 h. "+": EPS (slime) production after 48 h. "-": no EPS (slime) production.

All of the strains that tested positive for EPS production on CA + S were then grown on CA + S − YE and CA + S + YE for quantification of EPS produced by all of the individual isolates.

In the presence of only $CaCO_3$ without any sucrose supplementation, there was no EPS production. *Limosilactobacillus fermentum*, *L. plantarum*, and *L. reuteri* produced EPS after 24 h of incubation at 30 °C with 5% CO_2 on CA + S, CA + S − YE, and CA + S + YE. However, *L. kunkeei* produced EPS after 48 h with 5% CO_2 upon incubation at 30 °C. *L. fusant* did not produce EPS on any of the three agars.

Chalmers agar supplemented with 5% sucrose and without $CaCO_3$ is the most suitable medium for the growth of EPS-producing LAB, which can be visualized in the form of slimy colonies. EPS production depends on the medium's composition (i.e., nitrogen and carbon source and growth factors) and the temperature, pH, incubation time, and available oxygen to which the microorganisms are exposed. All LAB, AAB, and yeast showed growth on CA + S − $CaCO_3$, CA + S − YE, and CA + S + YE agar. Homopolysaccharide-producing LAB strains, such as *Lactobacillus reuteri*, often originate from cereal products, and heteropolysaccharide-producing LAB strains are present in high proportions in dairy products, which are fermented [63–65]. Synthesis of homopolysaccharide is correlated to the presence of sucrose in the media, which is the sole substrate for utilization by glycansucrase, while, on the other hand, heteropolysaccharide production is independent of the sucrose source [66].

Acetobacter aceti and *Acetobacter pasteurianus* produced EPS after 48 h of incubation at 30 °C with 5% CO_2 on CA + S − $CaCO_3$, CA + S − YE, and CA + S + YE agar (Table 5). No EPS was produced by *A. lovaniensis*. AAB are Gram-negative obligate aerobes and belong to the subdivision of α-proteobacteria (well-known vinegar producers). Almost all AAB species can grow in static culture by "floating" as they produce a pellicle on the surface of the culture medium. This pellicle is an aggregate of all of the cells suspended in the liquid–air interface and strongly bound to one another by polysaccharide or other extracellular matrices on the AAB cell surface. The pellicle polysaccharides occur as a homopolysaccharide of cellulose or as heteropolysaccharides, such as a capsular polysaccharide of *Acetobacter aceti* strain IFO3284. The *Acetobacter pasteurianus* strain produces two different types of colonies on the agar surface: a rough-surfaced colony and a smooth-surfaced colony. The rough-surfaced-colony-producer strain can produce pellicles, which allows it to float on the medium surface in static culture [67].

Regarding acetic acid bacteria, conflicting findings have emerged regarding the impact of carbon sources on EPS (extracellular polysaccharides) production. Studies indicate that fructose, sucrose, and glucose are associated with the highest yields of bacterial cellulose [68–72]. Yet, there remains a lack of data concerning how carbon sources affect the chemical composition of heteropolysaccharides like acetan or gluconacetan, which are synthesized by Acetobacter strains [73].

Candida guilliermondii and *Rhodotorula mucilaginosa* were positive for EPS production after 48 h of incubation at 28 °C for all CA + S, CA + S − YE, and CA + S + YE, whereas *C. kefyr*, *S. cerevisae*, and *C. colliculosa* were negative for phenotypic EPS production (Table 5).

Most of the Candida species can form surface-attached microbial communities by producing extracellular polymeric substances [74]. Gientka et al. (2016) [75] have reported the production of EPS by *Candida guilliermondii* isolated from kefir. A study carried out by Garza et al. (2016) [76] has reported that EPS is produced by *Rhodotorula mucilaginosa*. In most cases, EPS is produced when microorganisms are under conditions of stress and create a shell-like structure that prevents toxic reagents from reaching the cell [76–79].

3.5. EPS Quantification

All of the isolates that tested positive for EPS production on CA + S − YE and CA + S + YE agar (Table 5) were further quantified for EPS production as polymer dry mass in mg/kg. All of the LAB, AAB, and yeast strains show higher EPS production on Chalmers agar supplemented with 5% sucrose and 0.5% YE compared to CA + 5% sucrose without YE (Table 6).

Table 6. Quantification of EPS produced by LAB, AAB, and yeast isolates. Letters a–e represent significant EPS production by LAB, AAB, and yeast within the column.

Isolate	Chalmers Agar + 5% Sucrose and No Yeast Extract (Polymer Dry Mass in mg/kg)	Chalmers Agar + 5% Sucrose + 0.5% wt/vol Yeast Extract (Polymer Dry Mass in mg/kg)
Limosilactobacillus fermentum	87.89 ± 1.1 a	94.01 ± 1.6 a
Lactiplantibacillus plantarum	70.36 ± 0.9 b	85.21 ± 2.3 b
Lactobacillus reuteri	69.31 ± 1.8 b	87.92 ± 2.0 b
Lactobacillus kunkeei	29.33 ± 0.8 c	35.16 ± 1.8 c
Acetobacter aceti	25.7 ± 0.6 d	32.27 ± 0.9 c,d
Acetobacter pasteurianus	20.5 ± 0.6 e	24.55 ± 1.9 e
Candida guilliermondii	19.97 ± 0.6 e	29.66 ± 1.4 d
Rhodotorula mucilaginosa	30.92 ± 0.6 c	34.73 ± 1.5 c

Three out of the four LAB strains showed the significantly highest EPS production on CA + S + YE, between 85.21 ± 2.35 mg/kg and 94.01 ± 1.65 mg/kg polymer dry mass (PDM) ($p \leq 0.05$), when compared to EPS production by AAB and yeast.

The assessment of EPS (extracellular polysaccharides) in LAB (lactic acid bacteria) is commonly conducted using broth medium, which may be modified with various simple sugars, along with complex media. Hence, this study adopted a straightforward and beneficial approach. Another rationale behind employing a solid medium was to minimize the risk of contamination by polysaccharides, including certain proteins or other large molecules found in liquid culture mediums. Additionally, the solid surfaces of agar culture media encourage attached bacteria to produce EPS compared to planktonic cells in broth media [44]. *Acetobacter aceti* and *Acetobacter pasteurianus* produced higher EPS on CA + S + YE of 24.55 ± 1.99 and 32.27 ± 0.98 mg/kg PDM, respectively ($p \leq 0.05$). The genus Acetobacter is known for its capacity to produce cellulose (a type of EPS), which is used for some of the fermented food products [80–83]. A study carried out by Matsushita, Ebisuya, Ameyama, and Adachi (1992) provides evidence that *A. aceti* (R-strain or rough-surfaced-colony) produces EPS; however, no quantitative data were presented [84].

Candida guilliermondii and *Rhodotorula mucilaginosa* produced 29.66 ± 1.44 and 34.73 ± 1.58 mg/kg PDM, respectively ($p \leq 0.05$). Vazquez-Rodriguez et al. (2018) have reported that *Rhodotorula mucilaginosa* strain UANL-001 L produced 19 mg/mL of EPS after 96 h of incubation at 28 °C [80]. A study carried out by Gientka et al. (2016) shows that *Candida guilliermondii* was able to synthesize 2.9 g/L of EPS after 72 h of incubation and with maltose as the carbon source [75].

4. Conclusions

The biological and enzymatic phytase assays have shown that *Limosilactobacillus fermentum* and *L. plantarum* exhibited the highest phytase production and phytate-hydrolyzing properties.

Similarly, these two isolates exhibited the highest EPS-producing capability.

Except for *Lactobacillus fusant*, *Lactobacillus reuteri*, and *Lactobacillus kunkeei*, all of the LAB produced high glutamic acid concentration. Production of glutamic acid was observed for AAB and yeast isolates, but it was significantly lower when compared with those of LAB isolates. Interestingly, among all of the yeast isolates, *Candida guilliermondii* had the significantly lowest glutamic-acid-producing capability.

This paper has demonstrated that the microorganisms associated with sourdough fermentation in this study possess functional properties that could improve the sourdough.

5. Future Recommendations

The aim of this study was to determine the functional properties of the microorganisms identified and characterized from kefir, CWK, and sourdough fermented with coconut water kefir (CWKS). In addition to identifying functional properties, such as glutamic

acid production, phytase production, phytic acid degradation, and exopolysaccharide production, the analyses to determine the ability of the microorganisms to produce bioactive compound, such as GABA (γ-aminobutyric acid), could also be analyzed. Tailoring the screening criteria for functional strains to specific fermented foods and investigating the roles of the isolated microorganisms in the fermentation processes are suggested for further exploration.

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Abbreviations: LAB, lactic acid bacteria; AAB, acetic acid bacteria; CWK, coconut water kefir; CWKS, fermented sourdough (CWKS); GABA, γ-aminobutyric acid; EPS, exopolysaccharide; MRS, De Man–Rogosa–Sharpe agar; MOP, M 3-[N-Morpholino] propane sulfonic acid; CA, Chalmers agar; S, sucrose; YE, yeast extract.

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