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Characterization of the microbial diversity in yacon spontaneous fermentation at 20 °C

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ABSTRACT

The prebiotic fructooligosaccharides content of yacon makes this root an attractive alternative for the supplementation of a variety of food products. The preservation of yacon by fermentation has been proposed as an alternative to increase the probiotic content of the root concomitantly with its shelf life. Thus the fermented yacon could have significant functional content. The objective of this research was to characterize the biochemistry and microbiology of spontaneous yacon fermentation with 2% NaCl and define the viability of the proposed process. The biochemical analysis of spontaneous heterolactic fermentation of yacon showed a progressive drop in pH with increased lactic and acetic acids, and the production of mannitol during fermentation. The microbial ecology of yacon fermentation was investigated using culture-dependent and culture-independent methods. Bacterial cell counts revealed a dominance of lactic acid bacteria (LAB) over yeasts, which were also present during the first 2 d of the fermentation. Results showed that the heterofermentative LAB were primarily *Leuconostoc* species, thus it presents a viable method to achieve long term preservation of this root.

Key words: fermented vegetables, lactic acid bacteria, heterolactic fermentation.

1. Introduction

Fermentation of fruits and vegetables is one of the oldest technologies known to man. It results in the improvement of quality and safety of foods, due to the microbial biosynthesis of vitamins and essential amino acids, and the preservation of ascorbic acid, phenolic compounds and pigments (Galvez et al., 2007). Foods to be fermented carry a microbial load dependent on the soil, water, air and type of produce, with reported values of 10^3-10^5 CFU/g total aerobes (Rodríguez et al., 2009). The type of microorganisms and their development are determining factors of the course of the fermentation and the quality of the final product (Rodríguez et al., 2009). Typically, vegetable fermentations rely on spontaneous growth of indigenous lactic acid bacteria (LAB). Relevant determinants of the course of vegetable fermentations include temperature, initial pH, nutrients, and salt (NaCl) concentration in the cover brine solutions (Fleming et al., 1995). The concentration of NaCl used in vegetable fermentations ranges from 2% to 10% or more, depending on the type of products manufactured. NaCl favors the growth of LAB which produce lactic acid consequently dropping the medium pH and inhibiting the proliferation of competing undesirable bacteria. LAB are widely used in the food industry in the production of enzymes and metabolites, nutraceuticals, as vehicles of vaccines, as starter cultures in controlled fermentations, and as probiotics (Rodríguez et al., 2009). Although a large number of LAB starters are routinely used in dairy, meat and bakery fermentation, only a few cultures have been characterized and used at commercial scale for vegetable fermentations and none have been applied to yacon fermentation.

Yacon (*Smallanthus sonchifolius* Poepp.Endl) is an Andean root with known medicinal properties. The root has a sweet taste and crisp texture and is consumed raw, boiled, baked or as

juice. Yacon is now considered a functional food due to its fructooligosaccharides (FOS) and polyphenol contents (Flores et al., 2003; Genta et al., 2009; Ojansivu et al., 2010). Many yacon-based new products have been developed during the last 10 years, such as flour, syrup, jam, chips, and pickles among others (Reina et al., 2008; Ribeiro, 2008). The yacon, rich in FOS, reducing sugars, polyphenols, amino acids and minerals, has a considerable potential to make value-added products. Although, previous studies have shown that LAB are involved in yacon fermentation, the specific species involved are still unknown (Reina et al., 2009). As in many other vegetable fermentations (Ballesteros et al., 1999), the microbial diversity of yacon fermentation is significantly influenced by the amount of salt added. While yeasts predominate in salt-free yacon fermentation, heterofermentative and homofermentative LAB predominate in fermentations with 2-4% and more than 5% salt, respectively (Reina et al., 2009).

The objective of this study was to characterize and identify the microbial diversity of yacon spontaneous fermentation in the presence of 2% salt, using culture-dependent and independent methods. The fermentation of yacon is expected to preserve its nutritional value long term. A non-alcoholic fermentation is expected to impart an acceptable flavor and quality, while ensuring the safety of the good for human consumption. A fermentation lead by heterofermentative LAB has the potential to add value to the processed good, naturally rich in nutrients, by producing prebiotic exopolyssacharides from the indigenous FOS. Several heterofermentative LAB are able to produce mannitol and exopolysaccharides (EPS) from fructose/glucose mixtures. Even though these compounds are not produced industrially by fermentation, the prospect of producing them using a food grade LAB is promising (Saha and Racine, 2011). The production of mannitol and EPS in food by LAB could result in the manufacture of food products with an added nutritional value and use as functional foods.

2. Material and methods

2.1. Yacon

The first lot of yacon processed was harvested during the month of August and was obtained from Corvallis, Oregon, USA after storage at 4 °C. The lot of yacon obtained for the second trial was harvested in March and delivered to Raleigh, North Carolina from Dixon, California after 2 weeks storage at 4 °C. Roots with no physical damage and equal consistency to the touch were selected, peeled (Butler and Rivera, 2004) and processed as described by Reina et al. (2009), with some modifications. Briefly, approximately 20 lbs. of yacon were blanched at 95 ºC for 3 min to inactivate polyphenol oxidase and immediately cooled in the same amount (V/W) of 4% cover brine (NaCl) at 4 ºC, so that the salt would equilibrate at 2% (Figure 1). The blanching step was introduced to reduce the number of indigenous yeasts and inactivate polyphenoloxidase (Narai-Kanayama et al., 2007). Samples were then vacuum-packed in sterile glass jars with lids and kept at 20 ± 2 ºC until use. Jars were closed with commercial lug caps fitted with a rubber septum to allow for inoculation and sampling of the jars with sterile syringes. The lids were heated in boiling water to soften the plastisol gasket and immediately applied to the filled jars. The 20 ± 2 ºC incubation temperature was chosen to mimic the conditions under which fresh yacon is stored in the native Andean communities where the temperatures vary between 18-22 °C. In our study the jars were stored in the dark. Each experiment consisted of 3 replicates. Two independent lots of yacon were tested.

2.2. Microbiological Analyses

Cover brine samples were aseptically collected on days 7, 14, 21 and 30; serially diluted in saline solution and plated on plate count agar (PCA, Difco Laboratories, Detroit, MI), violet-

red bile agar (VRBG, Difco Laboratories) supplemented with 1% glucose, Lactobacilli de Man Rogosa and Sharpe agar (MRS, Difco Laboratories) supplemented with 0.5 g/L of L-Cys (Sigma-Aldrich, St. Louis, MO), and yeast extract malt agar (YMA, Difco Laboratories) containing 250 mg/liter chlortetracycline and 250 mg/liter chloramphenicol (Sigma Aldrich) to enumerate the total aerobic microbiota, *Enterobacteriaceae*, LAB, and yeasts and molds, respectively. MRS plates were incubated in anaerobic jars at 30 and 37 ºC for 48-72 h. The MRS plates were supplemented with cysteine and incubated at 37 °C to promote growth of any *Bifidobacetrium* spp. that could have been present in the samples. YMA and PCA plates were incubated aerobically at 30 ºC; while VRBG plates were incubated at 37 ºC. Plating and plate counting were performed using a spiral plater (model 4000; Spiral Biotech, Norwood, MA) with an automated colony counter (Protos Plus; Microbiology International, Frederick, MD). Ten colonies were selected from MRS agar plates incubated at 30 °C from sampling days 0, 2, 7, 15, and 30 based on their morphologies and streaked on the same medium to obtain pure cultures. Frozen stocks of the pure cultures were prepared in MRS broth containing 20% glycerol (Sigma-Aldrich) and stored at -80 ºC.

2.3. Total Genomic DNA Extraction

A propidium monoazide (PMA, Biotium, Inc., Hayword, CA) treatment was applied to 1 mL of the homogenized and filtered yacon samples as described by Pan and Breidt (2007) prior to DNA extraction to eliminate the amplification of nucleic acid fragments derived from dead cells. Total genomic DNA was extracted using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA) following the manufacturer's instructions. DNA from isolated

colonies growing on MRS was extracted using the InstaGene Matrix DNA extraction kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions.

2.4. 16S rDNA sequencing

Partial 16S rDNA sequencing was performed using the total genomic DNA extracted from complex samples or the bacterial isolates chromosomal DNA. The PCR mix contained 2X master mix (Bio-Rad), chromosomal DNA (5-10 ng), and 0.6 µM of primers RBUP (5-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') (Wilson et al., 1990). The PCR cycle consisted of 4 min at 95 ºC followed by 30 cycles of 30 s at 95 ºC, 30 s at 61 ºC, and 30 s at 72 ºC, with a final extension step of 7 min at 72 ºC and stored at 4 ºC until used. PCR products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA) and sequenced by Eton Bioscience Inc. (Durham, NC). The sequences obtained were subjected to the basic local alignment search tool (BLAST) (Altschul et al., 1990) using the 16S ribosomal RNA sequence database to determine the identity of the strains.

2.5 Total DNA analysis by the Ibis Technology

Total genomic DNA extracted from samples collected on days 1, 2, 7 and 14 were amplified as described above and delivered to AthoGen (Carlsbad, CA) for a broad range bacterial rDNA analysis using their Ibis Technology for microbial screening. The Ibis Technology analysis starts with a multiplex amplification of the genomic DNA extracted from our samples. Amplicons are desalted and the mass and base composition of the samples determined by NMR. Results are processed using specific algorithms, working in synchrony with

a pre-designed database that generates a report of the most abundant species present in the processed samples.

2.6. Total DNA sequencing analysis using Ion Torrent

For Ion torrent sequencing the V1-V2 fragment of the 16S rDNA was amplified from total bacterial DNA isolated from each sample using the forward primers (5´-xxxxxxxxxx*AGAGTTTGATCCTGGCTCAG* - 3´) where the underlined sequence contains the barcodes consisting of 10 nucleotides and the sequence in italics contains the universal primer 8F. The reverse primer was 338R (5´-TGCTGCCTCCCGTAGGAGT-3´). PCR reactions contained 5-10 ng of DNA template, 2.5 units of HotStar Hi-fidelity DNA polymerase (Qiagen, Valencia, CA), 1x HotStar Hi-Fidelity PCR buffer containing dNTPs, and 0.6 µM of each primer. Reaction conditions consisted of an initial denaturation for 5 min at 94 ºC followed by 35 cycles of 94 ºC for 60 s, 57 ºC for 60 s, and 72 ºC for 60 s, and a final extension of 72 ºC for 10 min. Negative controls, not containing template, were amplified for all barcode-primer sets. The PCR products with approximately 400 nucleotides were confirmed and purified by gel electrophoresis in a E-gel system (Invitrogen, Life Technologies, Grand Island, NY). DNA concentrations were quantified using PicoGreen dsDNA reagent (Invitrogen-Life Technologies) on a 96-well plate reader and mixed at equimolar concentrations. All kits were used according to the respective manufacturers' instructions. Purified libraries were submitted to the UNC Microbiome Core Facility (Chapel Hill, NC) for sequencing on the Ion Torrent platform. Sequencing data was submitted to the National Center for Biotechnology Information (NCBI) under BioProject ID PRJNA242408.

2.7. Amplicon high-throughput sequencing data analysis

Raw Ion Torrent fastq files were demultiplexed, quality-filtered, and analyzed using QIIME (Caporaso et al., 2010). The 400-bp reads were truncated at any site if more than three sequential bases receiving a quality score of *<20*, and any read containing ambiguous base calls or barcode/primer errors were discarded, as were truncated reads. Operational Taxonomic Units (OTUs) were assigned using the QIIME implementation of UCLUST (Edgar, 2010), with a threshold of 97% pairwise identity, and representative sequences from each OTU selected for taxonomy assignment. Beta diversity estimates were calculated within QIIME using weighted and unweighted Unifrac distances (Lozupone and Knight, 2005) between samples at a depth of 989 sequences per sample. From these estimates, jackknifed principal coordinates were computed to compress dimensionality into two- and three-dimensional principal coordinate analysis plots. QIIME was also used to calculate alpha diversity on rarefied OTU tables to assess sampling depth coverage using observed species, Shannon and phylogenetic diversity *(PD)* metrics.

2.8. Chemical Analyses

pH measurements were done using an Acumet® Research 25 pH meter (Fischer Scientific, CA, USA) equipped with a gel-filled pencil-thin pH combination electrode (Fischer Scientific, PA, USA). pH measurements were done from the aseptically collected cover brine samples or the extract of the homogenized fresh or fermented yacon samples. Yacon samples (10g) were homogenized at maximum speed for 1 min (Laboratory Blender, Stomacher 400, Tekmar, Cincinnati, OH) using filtered bags. The filtrate was used for pH measurements.

Brine samples (2 mL) were withdrawn aseptically at 7, 14, 21 and 30 d of fermentation for analysis by high-performance liquid chromatography (HPLC). Organic acids, sugars, and ethanol concentrations were measured with a Thermo Separation Products HPLC (ThermoQuest Inc., San Jose, CA) system consisting of a P1000 pump, an SCM100 solvent degasser, an AS3000 autosampler, and a UV6000 diode array detector (ThermoQuest). An HPX-87H column, 300 by 7.8 mm (Bio-Rad) was used with a differential refractometer (Waters model 410 Millipore, Milford, MA) and a UV detector (UV6000LP, Thermo Separation Products, San Jose, CA) for detection of the analytes. Operating conditions of the system included a sample tray at 6 ºC, a column at 65 ºC and 0.03 N H_2SO_4 eluent at a flow rate of 0.8 mL/min. The UV6000 detector was set to 210 nm at a rate of 1 Hz for data collection and 2 Hz was used for refractive index data. ChromQuest version 4.1 chromatography software was used to control the system and analyze the data.

3. Results and Discussion

3.1. Culture-dependent analysis of the microbial diversity of yacon fermentation

Previous studies have shown the effect of different salt concentrations on the microbial diversity of yacon fermentation (Reina et al., 2009). Yacon fermentation is dominated by yeasts, reaching populations of $2x10^6$ CFU/g if NaCl free cover brine is used (Reina et al., 2009). Reina study shows that a yacon fermentation with 2% NaCl at equilibrium, is dominated by LAB (10^9 CFU/g) followed by yeasts that reached a population of 10^{1-2} CFU/g. The metabolic activity of LAB drop the pH of the yacon to 3.7, which is well below the 4.6 pH required by the safety guidelines. Fermentation occurs rapidly (24 h), and the resulting product is stable. These

observations coincide with results reported from the fermentation of other vegetables, in which 2% salt concentration favors the growth of LAB such as *Leuconostoc* and *Lactococcus*.

In this study the fresh yacon indigenous microbiota was found to be primarily composed of aerobic mesophilic bacteria (6.27 ±0.3 Log CFU/mL), *Enterobacteriaceae* (5.04 ± 0.6 Log CFU/mL) and some yeasts (3.24 ±0.28 Log CFU/mL). LAB composed the majority of the mesophilic population with initial counts of 5.11 ± 1 Log CFU/mL. The total mesophilic bacteria, primarily composed of lactic acid bacteria, increased with time, reaching close to 8 log CFU/mL after 3 d of fermentation with 2% NaCl (Figure 2 and 3). Although, LAB counts were slightly higher when the plates were incubated at 30 ºC as compared to 37 ºC the differences in counts were not significant. The total number of *Enterobacteriaceae* decreased after 48 h of fermentation reaching numbers below the limits of detection as the pH decreased (Figure 2 and 5).

Yeasts were initially present in low numbers (Figure 2) and decreased below the limit of detection by the second day of the fermentation. An amplicon for the 18S rDNA using total eukaryotic DNA isolated from the yacon fermentation samples could not be obtained (data not shown). Together these data suggest that the blanching step and supplementation of the cover brine with 4% salt were sufficient to prevent the prevalence of yeasts in the process. Molds were not detected in the fresh yacon samples.

Colonies of LAB (39 total) from representative MRS plates at different points of fermentation were selected for further study. Table 2 shows the colonies identification using partial sequencing of the 16S rDNA. Three *Leuconostoc* spp. dominated the fermentation, *mesenteroides, pseudomesenteroides* and *citreum*. *Staphylococcus warneri* was detected in the fresh yacon only.

3.2. Non-culture-dependent analysis of the microbial diversity of yacon fermentation

Figure 4 presents the results of the sequencing analysis by Ion Torrent, which are in agreement with the data from the cultured based method and the Ibis Technology analysis. The Ion Torrent analysis confirmed that the dominant bacterial community in yacon fermentations are *Leuconostoc* species, including *L. mesenteroides*. This analysis additionally suggest that the community of Lactobacillales is dominated by *Leuconostoc* species, presumably due to the low percentage of salt added in the cover brine (2%) as described bothers for fermented vegetable products (Eom et. al., 2007; Jung et. al., 2012; Tamang et. al., 2005). The Ibis Technology broad bacterial analysis confirmed that *Leuconsotoc mesenteroides* was the dominant bacterium in yacon fermentation during the first two weeks (Table 2). Species of *Leuconostoc* are commonly found in fruits and vegetables and they play important roles in the fermentation of dairy products, vegetables and meats given their ability to ferment sugars (sucrose, glucose, fructose) producing lactic acid, glucans, fructans, and mannitol (Groben et al., 2001; Kim et al., 2002; Patra et al., 2011).

3.3. Biochemical characteristics of yacon fermentation

The fresh yacon had an initial pH of 5.77 ± 0.3, which decreased gradually with time to reach a final pH of 3.71 ± 0.11 after 7 d of fermentation (Figure 5). This reduction of pH coincided with the growth of LAB during the fermentation (Figure 2). Cover brine and yacon pH reached an equilibrium after 15 d of fermentation (figure 5). The use of small pre-cut pieces of the fruit may allow for a faster equilibration, which would likely induce a quicker suppression of

the undesired enterobacteriaceae population. Thus the use of small pre-cut pieces of fresh yacon should be considered as the best choice for the manufacture of a safer fermented good.

To assess the overall metabolic activity of the microbial community, the concentrations of sucrose, glucose, fructose, and organic acids were measured by HPLC (Table 3). The results suggest that yacon is a rich source of glucose, fructose and sucrose, and that these sugars were utilized as the carbon sources for the production of acetic and lactic acids and mannitol. These metabolites, in particular mannitol, represent those typically produced by heterofermentative *L. mesenteroides* in natural fermentations (Saha and Racine, 2011). The fluctuations in the amount of sugars detected during the fermentation are an indication of the lack of equilibrium in the system at the beginning of the fermentation, followed by a period of continuous equilibration and microbiological removal/utilization. Final concentrations of sugars are considered residual. Relatively high fructose concentrations were detected in some samples, suggesting the production of this sugar from the acid hydrolysis of FOS during the post-harvest curing (Grobben and others, 2001) or during the fermentation (Narai-Kanayama et al., 2007; Reina et al., 2009).

To our knowledge this is the first study showing the identification of LAB in yacon fermentation. Our results show the prevalence of heterofermtative LAB, mainly *Leuconostoc* in the indigenous yacon fermentation with 2% NaCl. Among the *Leuconostoc* spp. identified, one species, *mesenteroides*, dominated across the three techniques used for microbial identification. Utilization of a starter culture of *L. mesenteroides* or a combination of *Leuconostoc* spp. and of small pre-cut pieces of yacon may be advantageous in yacon fermentation with 2% salt to accelerate acid production and the equilibration of the system, respectively. Such strategy should

result in the quicker reduction of the undesired and indigenous *Enterobacteriacea* and minimized their proliferation.

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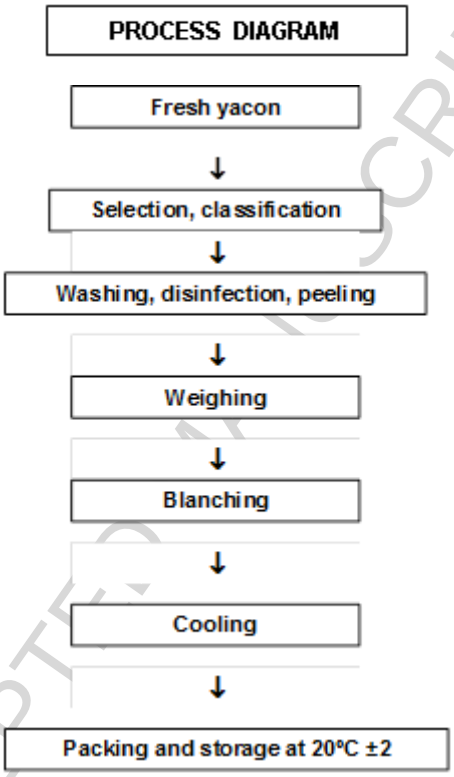
Figure 1: Flow chart to prepare pickled yacon for fermentation (a) and yacon packed with cover brine (b).

(a)



(b)

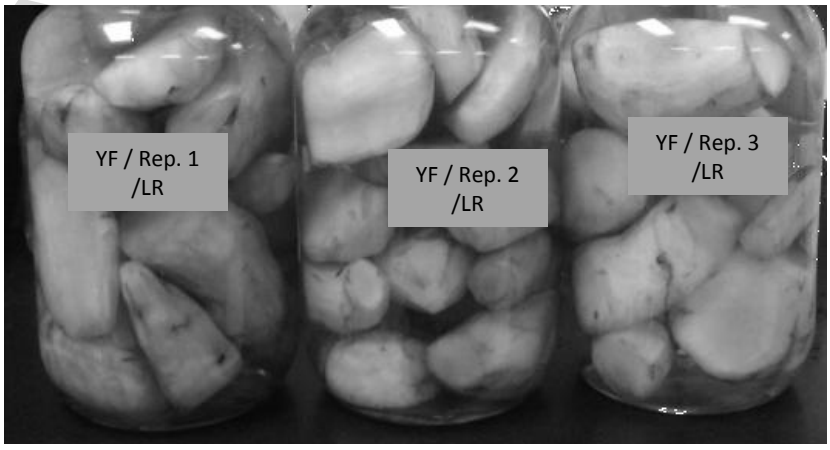


Figure 2: Microbial counts during yacon fermentation. Total aerobic counts from PCA (♦), counts for enterobacteriacea from VRBG (▲) and counts for yeasts from YMA (■) represent the average of triplicate samples with standard errors. Counts from YMA were below the detection levels after day 1. Minimum detection level was 2.4 Log CFU/mL. Fermentation age axis is not presented to scale.

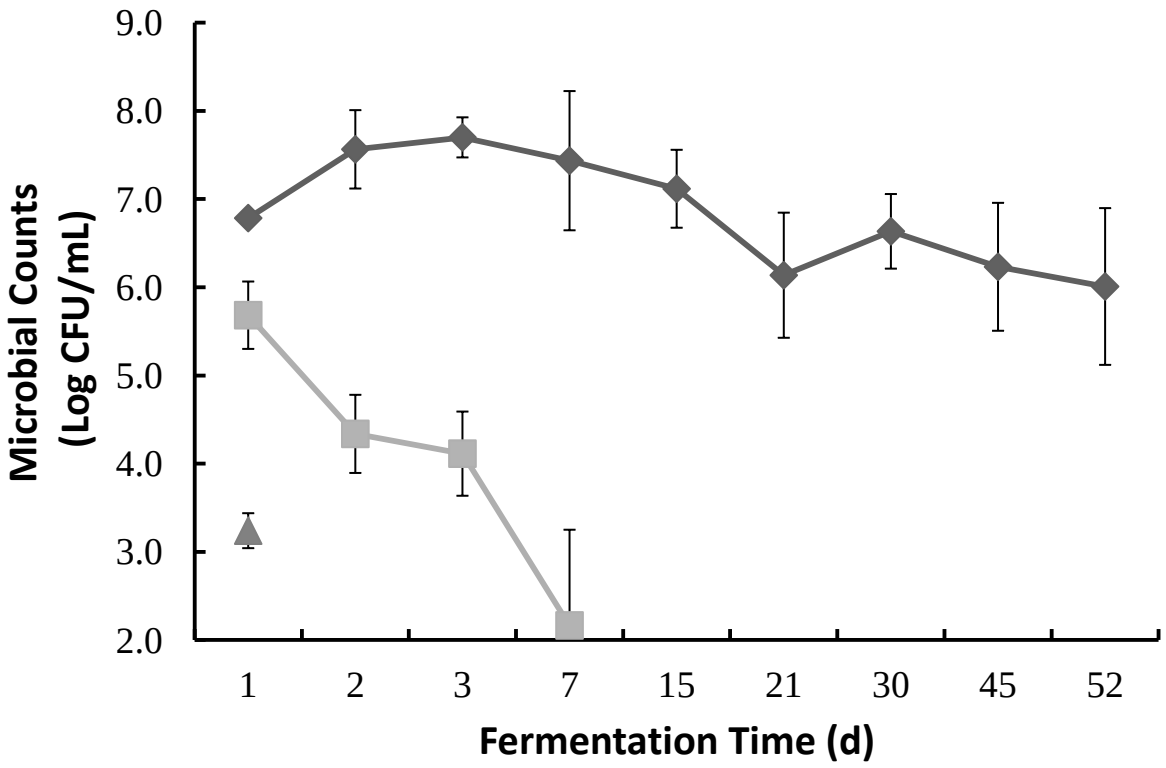


Figure 3: Lactic acid bacteria counts during yacon fermentation. Counts obtained from MRS agar plates incubated at 30 °C (♦) and 37 °C (■) under anaerobic conditions, represent the average of triplicate samples with standard error. Fermentation age axis was not drawn to scale.

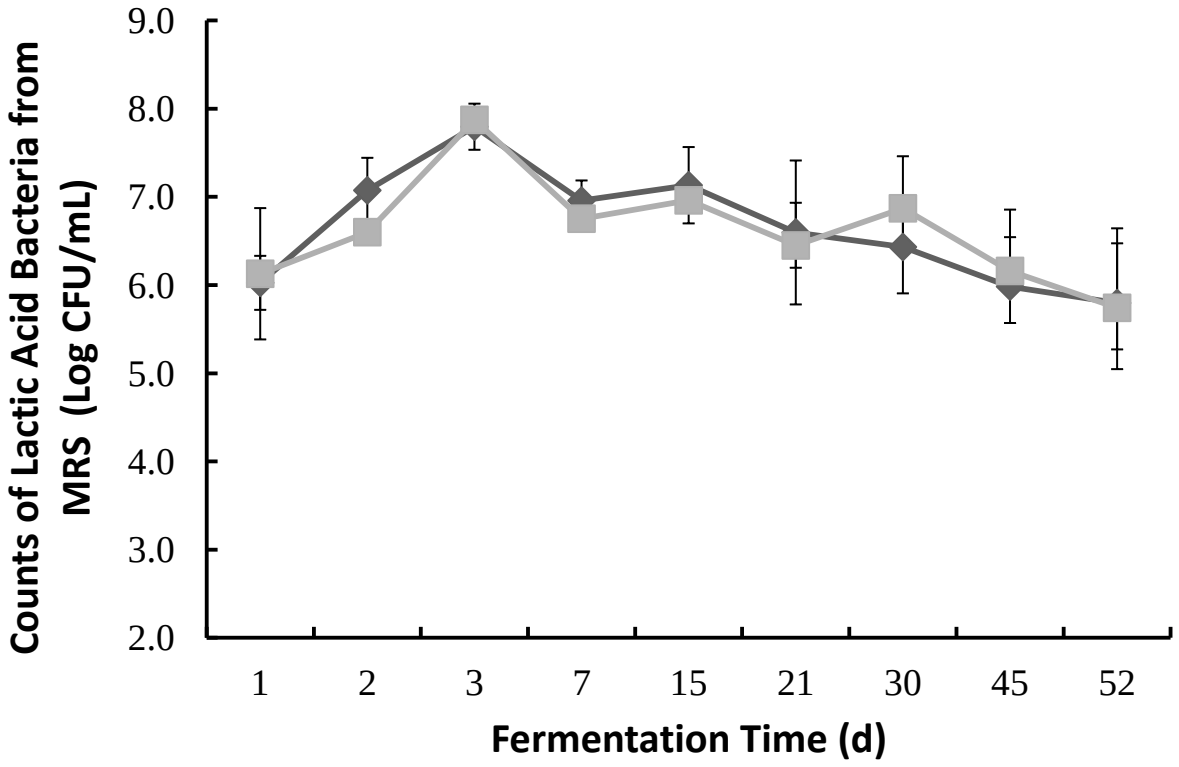


Figure 4: Distribution of bacterial species detected by total genome sequencing using the Ion Torrent technology at the beginning and the peak of a yacon fermentation.

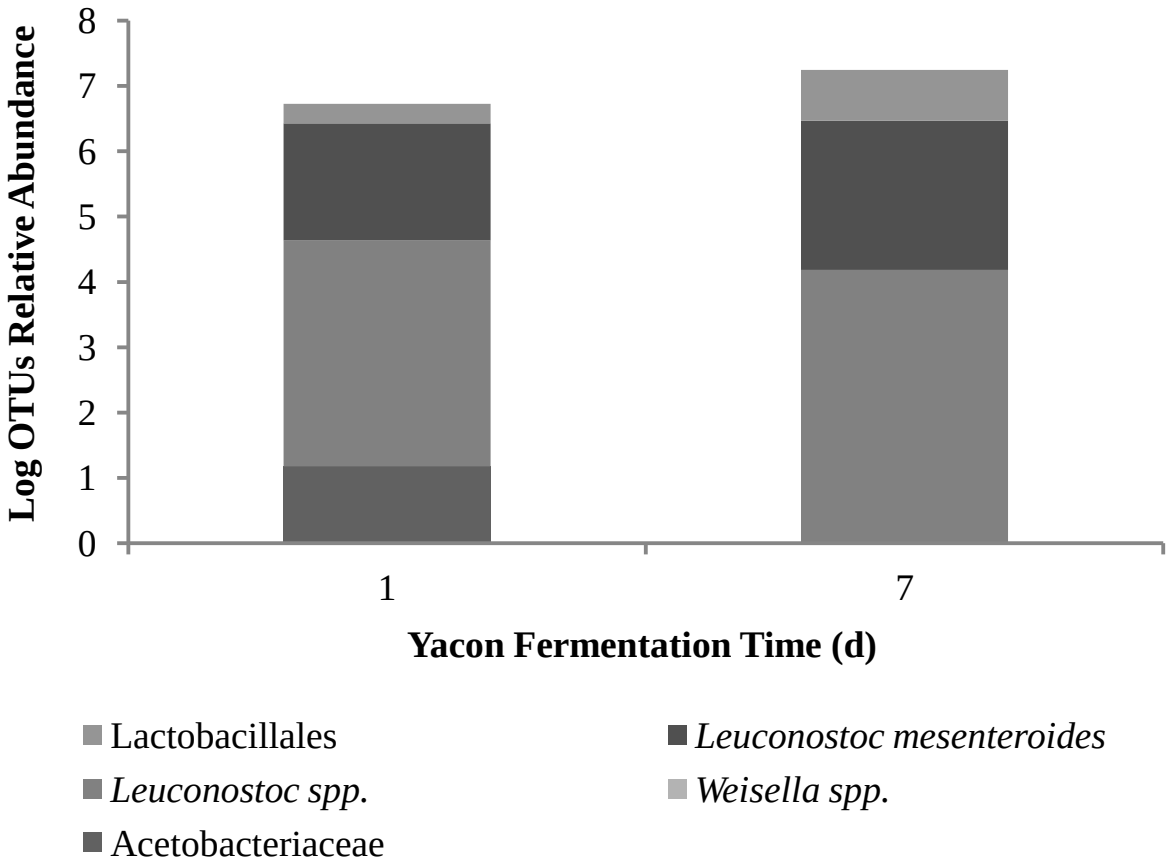


Figure 5: Changes of pH during yacon fermentation. Numbers presented for the cover brine samples (▲) are the averages of triplicate from two independent replicates. Numbers presented for the brined yacon samples (♦) represent triplicates of one lot of yacon and standard deviations. The fermentation age axis is not drawn to scale.

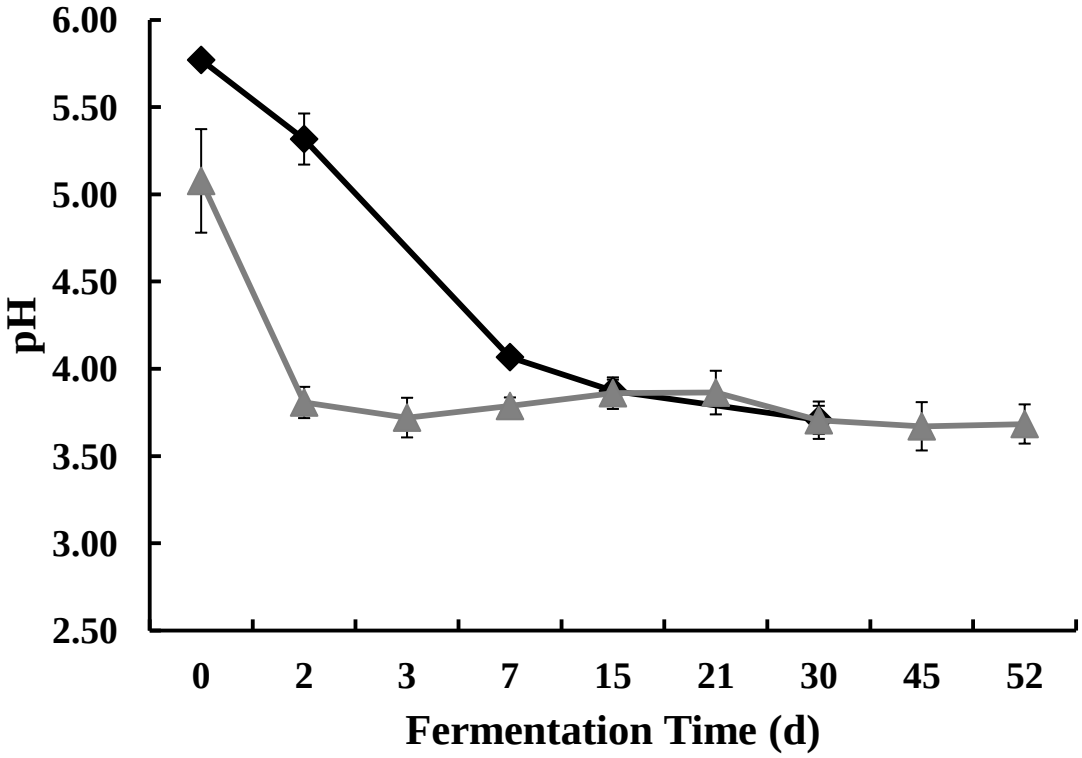


Table 1. Identity of cultivated bacteria from yacon fermentation as determined by the partial sequencing of the 16S rDNA

Fermentation Time (d)	Best BLAST Hits	GeneBank Accession No. for Representative Sequences	No. of Colonies Identified
0 (fresh)	*Staphylococcus warneri*	KF661311	2
2	*Leuconostoc mesenteroides*	KF661314	6
7	*Leuconostoc mesenteroides* *Leuconostoc pseudomesenteroides*	KF661315 KF661313	6 1
15	*Leuconostoc mesenteroides* *Leuconostoc pseudomesenteroides*	KF661302, KF661308 KF661309	7 1
30	*Leuconostoc mesenteroides* *Leuconostoc citreum*	KF661319, KF661320 KF661310	12 1

Table 2. Culture independent detection of predominant bacteria using PCR-NMR as reported by AthoGen

Fermentation Time (d)	Quality Score	Amplicon Identifications
1	0.99	*Leuconostoc mesenteroides*
2	0.99	*Leuconostoc mesenteroides*
7	0.99	*Leuconostoc gasicomitatum/kimchii/pseudomesenteroides*/sp.
15	0.99	*Leuconostoc mesenteroides*
	0.97	*Enterobacter* sp.

Table 3. Biochemical changes during yacon spontaneous fermentation. Values presented are the average of triplicate samples with standard deviations.

Fermentation Age (days)	Components Detected by HPLC Analysis (mM)								
	Glucose	Fructose	Sucrose	Malic Acid	Succinic Acid	Fumaric Acid	Lactic Acid	Acetic Acid	Mannitol
0	33.1 ± 0.5	123.7 ± 1.4	2.1 ± 0.4	9.5 ± 0.2	9.0 ± 0.1	6.3 ± 0.1	ND	ND	ND
2	75.1 ± 1.1	299.9 ± 3.5	9.2 ± 0.6	22.8 ± 0.3	27.3 ± 0.2	20.3 ± 0.2	ND	ND	ND
7	12.3 ± 6.1	55.3 ± 9.5	5.8 ± 5.0	3.8 ± 1.5	2.9 ± 0.4	ND	3.5 ± 2.7	2.6 ± 4.6	ND
15	4.3 ± 1.2	35.8 ± 8.1	7.3 ± 0.3	1.8 ± 1.0	1.5 ± 0.3	ND	2.5 ± 0.5	4.6 ± 8.0	ND
30	4.6 ± 4.1	ND*	5.0 ± 4.3	2.0 ± 1.6	2.1 ± 1.9	ND	19.6 ± 7.7	42.1 ± 1.9	105.7 ± 6.5

*ND: not detected

Highlights

- Lactic acid bacteria predominate in yacon fermentation with 2% salt.
- Sugars from yacon are microbiologically converted to organic acids and mannitol.
- *Leuconostoc* spp. lead yacon fermentation with 2% salt.
- Yacon can be preserved long term by fermentation with 2% salt.