



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



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ABSTRACT

The prebiotic fructooligosaccharide 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 days 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.

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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 a 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 fructooligosaccharide (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.,

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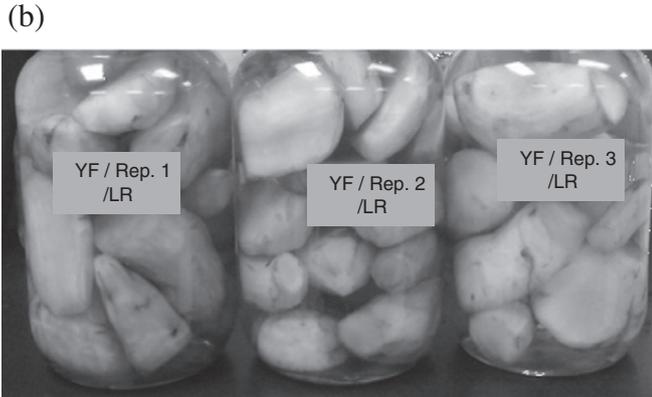
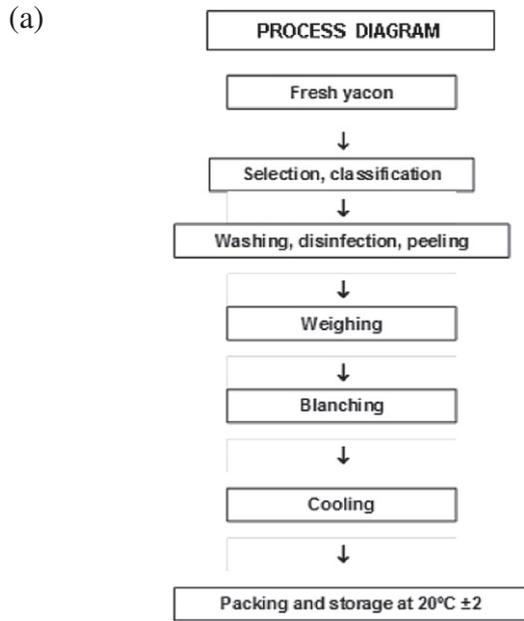


Fig. 1. Flow chart to prepare pickled yacon for fermentation (a) and yacon packed with cover brine (b).

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 led by heterofermentative LAB has the potential to add value to the processed good, naturally rich in nutrients, by producing prebiotic exopolysaccharides 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.

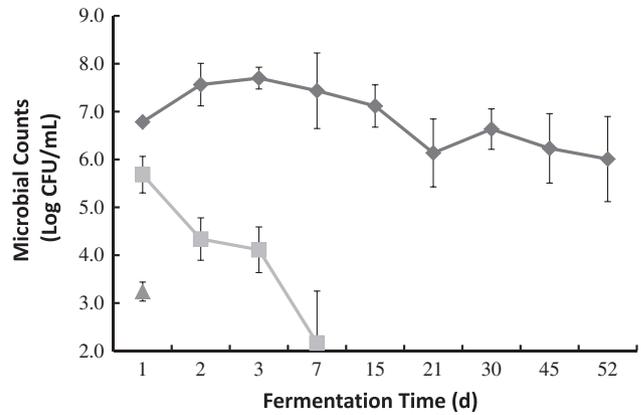


Fig. 2. Microbial counts during yacon fermentation. Total aerobic counts from PCA (◆), counts for *Enterobacteriaceae* from VRBG (▲) and counts for yeasts from YMA (■) represent the average of triplicate samples with standard errors. Counts from YMA were below the detection levels after day 1. Minimum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

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 of 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 was blanched at 95 °C for 3 min to inactivate polyphenol oxidase and immediately cooled in the same amount (V/W) of 4% cover brine (NaCl) at 4 °C, so that the salt would equilibrate at 2% (Fig. 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 °C 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 °C incubation temperature was chosen to mimic the conditions under which fresh yacon is stored in the native Andean communities

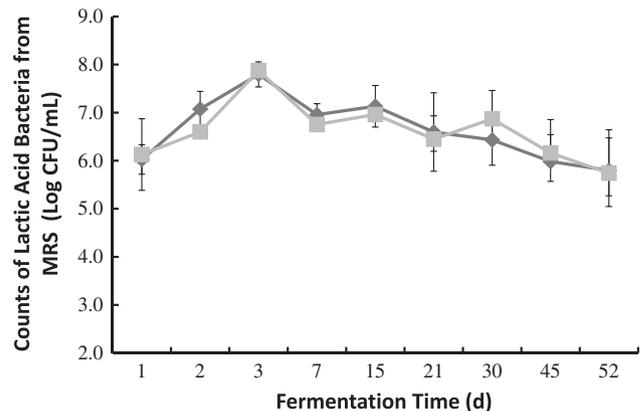


Fig. 3. Lactic acid bacteria counts during yacon fermentation. Counts obtained from MRS agar plates incubated at 30 °C (◆) and 37 °C (■) under anaerobic conditions, represent the average of triplicate samples with standard error. Fermentation age axis was not drawn to scale.

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

Fermentation time (days)	Best BLAST hits	GeneBank accession no. for representative sequences	No. of colonies identified
0 (fresh)	<i>Staphylococcus warneri</i>	KF661311	2
2	<i>Leuconostoc mesenteroides</i>	KF661314	6
7	<i>Leuconostoc mesenteroides</i>	KF661315	6
	<i>Leuconostoc pseudomesenteroides</i>	KF661313	1
15	<i>Leuconostoc mesenteroides</i>	KF661302, KF661308	7
	<i>Leuconostoc pseudomesenteroides</i>	KF661309	1
30	<i>Leuconostoc mesenteroides</i>	KF661319, KF661320	12
	<i>Leuconostoc citreum</i>	KF661310	1

where the temperatures vary between 18 and 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/L chlortetracycline and 250 mg/L 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 °C for 48–72 h. The MRS plates were supplemented with cysteine and incubated at 37 °C to promote growth of any *Bifidobacterium* spp. that could have been present in the samples. YMA and PCA plates were incubated aerobically at 30 °C; while VRBG plates were incubated at 37 °C. 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 °C.

2.3. Total genomic DNA extraction

A propidium monoazide (PMA, Biotium, Inc., Hayward, 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

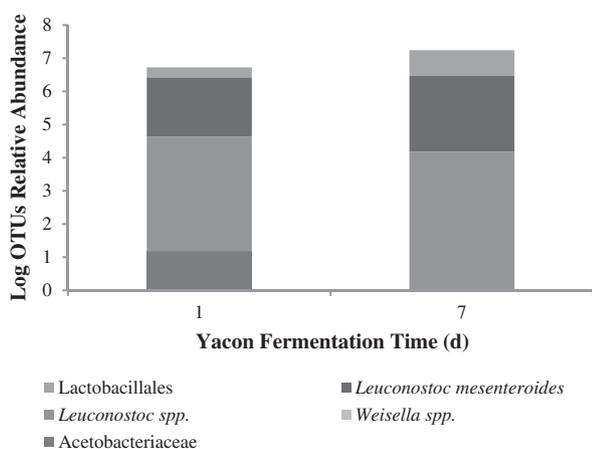


Fig. 4. Distribution of bacterial species detected by total genome sequencing using the Ion Torrent technology at the beginning and the peak of a yacon fermentation.

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 isolate chromosomal DNA. The PCR mix contained 2X master mix (Bio-Rad), chromosomal DNA (5–10 ng), and 0.6 μM of primers RBUP (5-AGAGTTTGAT CCTGGCTCAG-3') and 1492r (5'-GGTACCTTGTTACGACTT-3') (Wilson et al., 1990). The PCR cycle consisted of 4 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 61 °C, and 30 s at 72 °C, with a final extension step of 7 min at 72 °C and stored at 4 °C 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 was 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'-xxxxxxxxxxAGAGTTTGATCCTGGCTCAG-3') where the underlined sequence contains the barcodes consisting of 10

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

Fermentation time (days)	Quality score	Amplicon identifications
1	0.99	<i>Leuconostoc mesenteroides</i>
2	0.99	<i>Leuconostoc mesenteroides</i>
7	0.99	<i>Leuconostoc gasicomitatum/kimchii/pseudomesenteroides/sp.</i>
15	0.99	<i>Leuconostoc mesenteroides</i>
	0.97	<i>Enterobacter</i> sp.

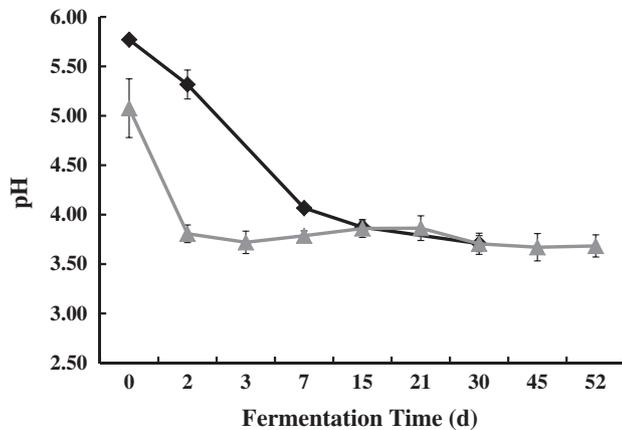


Fig. 5. Changes of pH during yacon fermentation. Numbers presented for the cover brine samples (▲) are the averages of triplicate from two independent replicates. Numbers presented for the brined yacon samples (◆) represent triplicates of one lot of yacon and standard deviations. The fermentation age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

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 μ M of each primer. Reaction conditions consisted of an initial denaturation for 5 min at 94 °C followed by 35 cycles of 94 °C for 60 s, 57 °C for 60 s, and 72 °C for 60 s, and a final extension of 72 °C 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 an 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 Accumet® Research 25 pH meter (Fisher Scientific, CA, USA) equipped with a gel-filled pencil-thin pH combination electrode (Fisher 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 (10 g) 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-days 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 °C, a column at 65 °C and 0.03 N H₂SO₄ 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 2×10^6 CFU/g if NaCl free cover brine is used (Reina et al., 2009). Reina's 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 drops 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

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 ^a	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

^a ND: not detected.

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 days of fermentation with 2% NaCl (Figs. 2 and 3). Although, LAB counts were slightly higher when the plates were incubated at 30 °C as compared to 37 °C 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 (Figs. 2 and 5).

Yeasts were initially present in low numbers (Fig. 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 1 shows the colonies identified 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

Fig. 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 is *Leuconostoc* species, including *Leuconostoc mesenteroides*. The Ion Torrent analysis additionally suggests that the community of Lactobacillales is dominated by *Leuconostoc* spp., presumably due to the low percentage of NaCl added in the cover brine (2%), as described by others for fermented vegetable products (Eom et al., 2007; Jung et al., 2012; Tamang et al., 2005). The Ibis Technology broad bacterial analysis confirmed that *L. 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 (Grobben 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 days of fermentation (Fig. 5). This reduction of pH coincided with the growth of LAB during the fermentation (Fig. 2). Cover brine and yacon pH reached equilibrium after 15 days of fermentation (Fig. 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 et al., 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 heterofermentative 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 *Enterobacteriaceae* and minimized their proliferation.

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