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ORIGINAL ARTICLE

Biodiversity and technological potential of lactic acid bacteria isolated from spontaneously fermented amaranth sourdough

L. Ruiz Rodríguez[1,†], E. Vera Pingitore[1,†], G. Rollan[1], G. Martos[1], L. Saavedra[1], C. Fontana[2], E.M. Hebert[1] and G. Vignolo[1]

1 Centro de Referencia para Lactobacilos (CERELA), CONICET, Tucumán, Argentina
2 Istituto di Microbiologia-Centro Ricerche Biotecnologiche, Università Cattolica del Sacro Cuore, Piacenza-Cremona, Italy

Significance and Impact of the Study: Nowadays, there is an increasing interest in ancient noncereal gluten-free (GF) crops such as amaranth, due to their reported nutritional and health benefits. However, the use of these grains is still limited to traditional foods and bread making processes that are not yet well standardized. Results on the dynamics of autochthonous lactic acid bacteria (LAB) microbiota during laboratory spontaneous amaranth sourdoughs (AS) fermentation will contribute to overcome challenges for GF-fermented products development. In addition, knowledge about LAB diversity involving *Enterococcus*, *Pediococcus* and *Lactobacillus* species, with *Lactobacillus plantarum* predominating during AS fermentation, and their technological and functional properties provides the basis for the selection of autochthonous strains as starters cultures for novel gluten-free bakery products with enhanced nutritional, sensory and/or safety quality.

Keywords
amaranth, functional foods, lactic acid bacteria, sourdough, starter culture.

Correspondence
Elvira M. Hebert, Chacabuco 145, (T4000ILC) San Miguel de Tucumán, Tucumán, Argentina.
E-mail: ehebert@cerela.org.ar

†These two authors contributed equally to this work.

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Abstract

Spontaneous fermented sourdoughs prepared from amaranth flour were investigated for the presence of autochthonous lactic acid bacteria (LAB) predominating microbiota. The doughs were fermented with daily backslopping on a laboratory scale at 30°C for 10 days. LAB counts ranged from 2·60 to 8·54 log CFU g^{-1} with a pH declined from 6·2 to 3·8 throughout fermentation. The combined use of randomly amplified polymorphic DNA (RAPD)-PCR analysis and sequence analysis of 16S rRNA was applied for LAB intraspecies differentiation and taxonomic identification, respectively. *Enterococcus*, *Pediococcus* and *Lactobacillus* species were present in amaranth sourdoughs (AS). After the first refreshment step, *Lactobacillus plantarum* dominated AS until the end of fermentation. In coincidence, when DGGE analysis was performed, the occurrence of a progressive change in bacterial communities allowed the selection of *Lact. plantarum* as a dominant species. Moreover, technological, functional and safety characteristics of representative RAPD-biotypes were investigated. *Lact. plantarum* CRL1898 was selected as a potential candidate for gluten-free amaranth sourdough starter.

Introduction

Amaranthus have long been cultivated in Central and South America for more than 5000–7000 years. There are about 60 different species, among which *Amaranthus caudatus* is the most cultivated in South American Andean region for food production. Starch (50–70%) and protein (13–15·5%) are among the main components of amaranth grains; amino acid composition reveals a rich source of lysine and methionine, which are critical for human nutrition (Rastogi and Shukla 2013). In addition to the high vitamin and mineral content, amaranth seeds and flour are potential source of bioactive peptides with biological functions beneficial to health (Silva-Sánchez *et al.* 2008; Caselato-Sousa and Amaya-Farfán 2012). Recently, amaranth human consumption has grown due

to favourable reports about amaranth health benefits and nutraceutical value (Rastogi and Shukla 2013). A major attraction is that Andean cereals are naturally gluten-free (GF) sources for formulations intended for celiac patients. Although gluten absence results in problems for bakers, recent interest on bakery products consisting completely or partially of GF flours gave rise to a number of novel GF food products (Gallagher *et al.* 2004; Coda *et al.* 2014).

Sourdough represents a natural food ecosystem in which the fermentation activities of lactic acid bacteria (LAB) and yeasts largely determine the typical characteristics of the resulting baked goods (Scheirlinck *et al.* 2007). The microbial composition of mature sourdoughs from various European countries using conventional cereal flours has been extensively investigated during the last years revealing a large LAB diversity (De Vuyst *et al.* 2014), whereas ancient and GF flours fermentation were less explored (Sterr *et al.* 2009; Ruiz Rodríguez *et al.* 2016). The prevalence of highly adapted and sourdough-specific LAB species was found to occur; the type and quality of flour used is of utmost importance as a source of nutrients, endogenous enzymes, and autochthonous LAB (De Vuyst *et al.* 2014). Following recent trends, food microbiology has benefited from the advances in molecular biology and adopted novel strategies to detect, identify, and monitor microbes in food. An in-depth study of the microbial diversity and population dynamics present in a specific ecosystem was achieved by using culture-independent methods. Among these, denaturing gradient gel electrophoresis (DGGE) and more recently high-throughput sequencing (HTS) have been applied (Cocolin *et al.* 2013; Ercolini 2013).

The exploitation of sourdough-resident LAB with technological, functional and safety competences will allow to design functional starter cultures for GF products with nutritional/health benefits (Coda *et al.* 2014). On these bases, the identification, population dynamics, technological-functional and safety characterization of autochthonous LAB from spontaneous fermentation of Andean amaranth flour were carried out.

Results and discussion

Microbiological counts, identification and dynamics of LAB from spontaneous fermented amaranth sourdoughs

Total mesophiles and LAB counts as well as pH values during amaranth sourdoughs (AS) fermentation are shown in Fig. 1. Fermentation was characterized by a rapid increase of LAB from the beginning of fermentation, major changes occurring within the first three days of propagation. LAB counts increased from 2·60 to 8·54 log CFU g^{-1}. The preponderance of LAB during AS fermentation was in correlation with pH values, which decreased from 6·2 to 3·8 at day 10. These results are correlated with those previously reported for amaranth and other GF spontaneously fermented flours (Sterr *et al.* 2009; Weckx *et al.* 2010; Ruiz Rodríguez *et al.* 2016).

Based on characterization by Gram staining and catalase activity, 91 isolates (18 cocci and 73 rods) were selected as presumptive LAB. All isolates were subjected to randomly amplified polymorphic DNA (RAPD)-PCR analysis by using the primers M13b and XD9. Strains showing identical RAPD-PCR patterns were considered as one RAPD-biotype. Thus, isolates were grouped into seven M13b and six XD9 different RAPD-biotypes (Fig. 2), and identified by partial 16S rRNA gene sequencing (Table 1). M13b RAPD-biotypes (M13b-Bt) (Fig. 2a; Table 1) were associated with *Enterococcus casseliflavus* (Bt1), *Ent. mundtii* (Bt2), *Lact. rhamnosus* (Bt3), *Pediococcus pentosaceus* (Bt4) and *Lact. plantarum* (Bt5, 6 and 7). On the other hand, when primer XD9 was used (Fig. 2b; Table 1), RAPD-biotypes were related to *Enterococcus* (Bt8, 9 and 10), *Ped. pentosaceus* (Bt11) and *Lact. plantarum* (Bt12 and 13). Remarkably, the use of RAPD-PCR for intraspecies discrimination allowed differentiating genotypically three strains of *Lact. plantarum,* highlighting the biodiversity among *Lact. plantarum* strains isolated. These results are consistent with those exposed by Pisano *et al.* (2010), who confirmed heterogeneous phenotypic traits of *Lact. plantarum* strains by using genotyping data obtained by RAPD-PCR profiles with M13 primer. Thus, RAPD-PCR was a useful approach to get information about biodiversity intra *Lact. plantarum* group. In addition to culture-dependent methods, PCR-DGGE was carried out to follow bacterial community dynamics in the sourdough ecosystem (Fig. 3; Table S1). PCR-DGGE analysis showed that major changes in bacterial community occurred at the beginning of fermentation (0–3 days) and only minor changes were observed subsequently. Bands 1, 2 and 6 from days 0 and 1 (*Klebsiella oxytoca*, *Enterobacter* sp. and *Escherichia blattae*) were only visible at these sampling points disappearing thereafter. During early stages of AS fermentation (days 1–3), two faint bands (3 and 5) were identified as *Enterococcus devriesei* and *Ent. mundtii* respectively, whereas a more intense band (4) revealed the presence of *Ent. faecium.* On days 2 and 3, *Ped. acidilactici* (band 8) was evidenced. Furthermore, three intense bands (7, 11 and 12) were identified as *Lact. plantarum*; their presence appeared from the second day up to the end of AS fermentation in agreement to results observed by culture-dependent methods. In addition, two more bands identified as uncultured *Clostridium* (9) and *Cyanobacterium* (10) were detected (Fig. 3). Based on these results, a

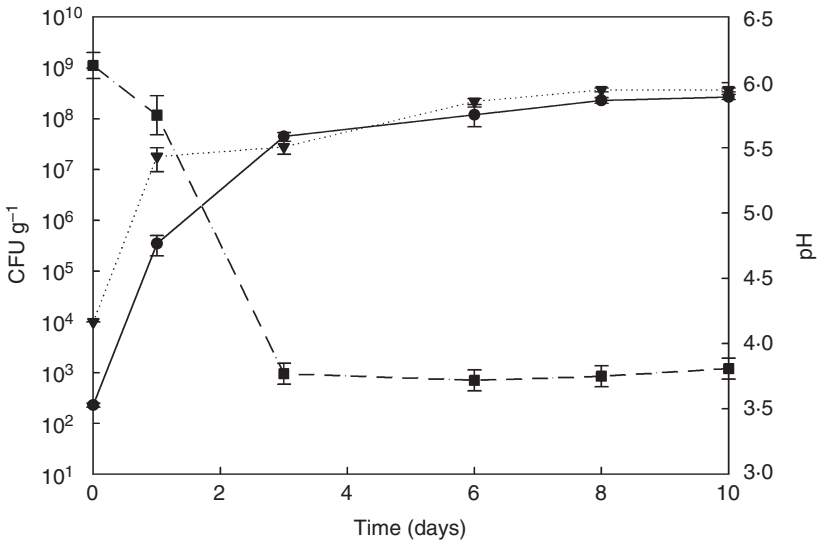


Figure 1 Changes in pH (■) and microbial counts of total bacteria (▼) and LAB (●) during spontaneous amaranth sourdoughs fermentation for 10 days at 30°C. Counts were performed in triplicate, and mean values ± standard deviations are presented.

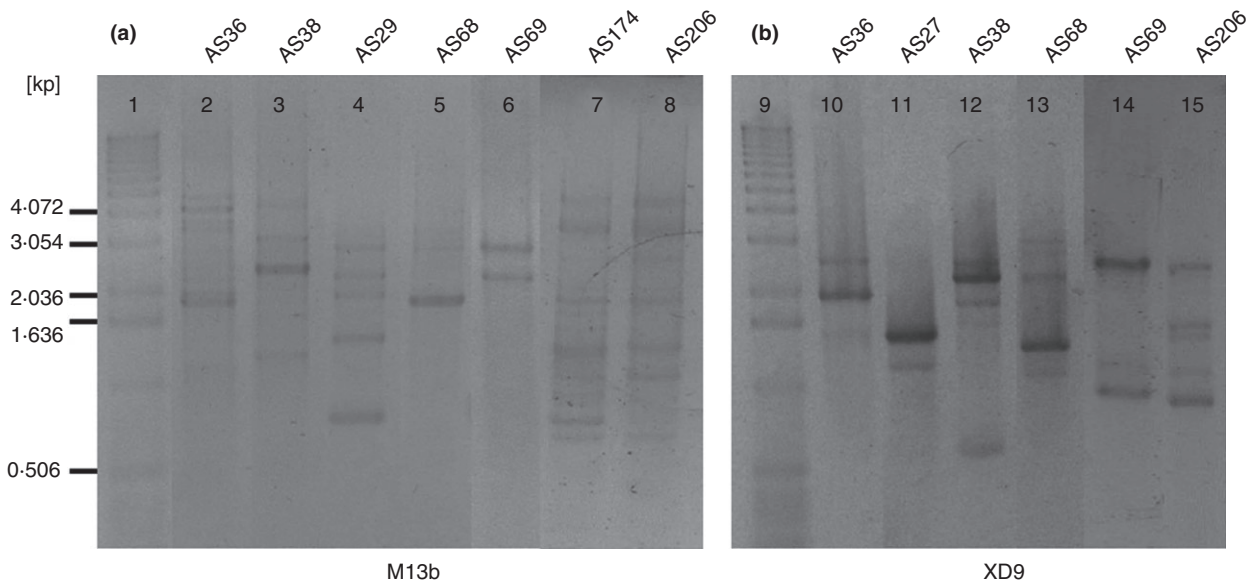


Figure 2 RAPD-PCR patterns of representative LAB isolates obtained with primers M13b (a) and XD9 (b) from different backslopping stages. Lines 1 and 9: molecular weight marker (1 kb DNA ladder, Invitrogen), 2: AS36 (*Enterococcus casseliflavus*, Bt1), 3: AS38 (*Enterococcus mundtii*, Bt2), 4: AS29 (*Lactobacillus rhamnosus*, Bt3), 5: AS68 (*Pediococcus pentosaceus*, Bt4), 6: AS69 (*Lactobacillus plantarum*, Bt5), 7: AS174 (*Lact. plantarum*, Bt6), 8: AS206 (*Lact. plantarum*, Bt7), 10: AS36 (*Ent. casseliflavus*, Bt8), 11: AS27 (*Ent. mundtii*, Bt9), 12: AS38 (*Ent. mundtii*, Bt10), 13: AS68 (*Ped. pentosaceus*, Bt11), 14: AS69 (*Lact. plantarum*, Bt12), 15: AS206 (*Lact. plantarum,* Bt13).

succession of LAB species with the persistence of *Lactobacillus* was found during AS fermentation either by the 16S rRNA analysis of the isolated strains and by PCR-DGGE analysis. However, the LAB microbiota obtained by culturing did not fully agree with the biodiversity data found through PCR-DGGE fingerprinting. By using culture-independent methodology, it was not able to retrieve neither *Ent. casseliflavus*, *Ped. pentosaceus* nor *Lact. rhamnosus* identified by culturing; conversely *Ent. devriesei*, *Ent. faecium* and *Ped. acidilactici* were detected only by PCR-DGGE analysis (Table 2). As reported by Carraro *et al.* (2011), a combined approach provides an overall picture of AS microbial ecology.

LAB species composition at each backslopping stage and dynamics indicated that a complex microbiota dominated by cocci species was initially present (days 0–2). The presence of *Ent. casseliflavus* and *Ent. mundtii* agree with those reported from wheat kernels, nonconventional flours and spontaneous rye and spelt sourdoughs (Corsetti *et al.* 2007; Weckx *et al.* 2010). In addition, two

Table 1 Lactic acid bacteria isolates biotypes and sequence information for RAPD-PCR obtained with primers M13b and XD9 during amaranth sourdough (AS) fermentation

Isolate	CRL No.*	Back-slopping day	Closest relative	RAPD-biotypes		Identity, %	Accession No.
				M13b	XD9		
AS27	1900	0	*Enterococcus mundtii*	–	9	99	KJ402409
AS36	1903	0	*Enterococcus casseliflavus*	1	8	99	KJ402412
AS38	1953	0	*Enterococcus mundtii*	2	10	97	KJ402411
AS29	1891	0	*Lactobacillus rhamnosus*	3	–	99	KJ402410
AS68	1902	1	*Pediococcus pentosaceus*	4	11	99	KJ402413
AS69	1898	1	*Lactobacillus plantarum*	5	12	99	KJ402414
AS174	1956	6	*Lactobacillus plantarum*	6	–	100	KF545927
AS206	1957	8	*Lactobacillus plantarum*	7	13	100	KJ402415

*Assigned number at the CERELA culture collection.

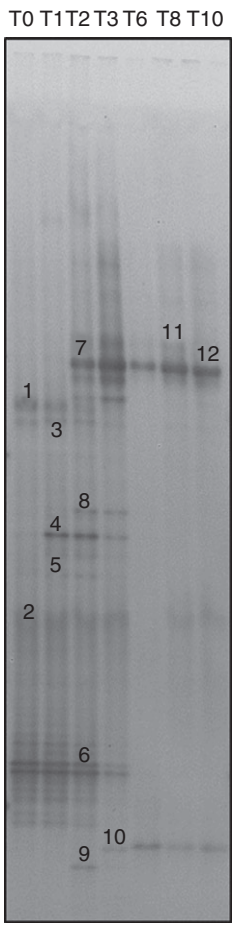
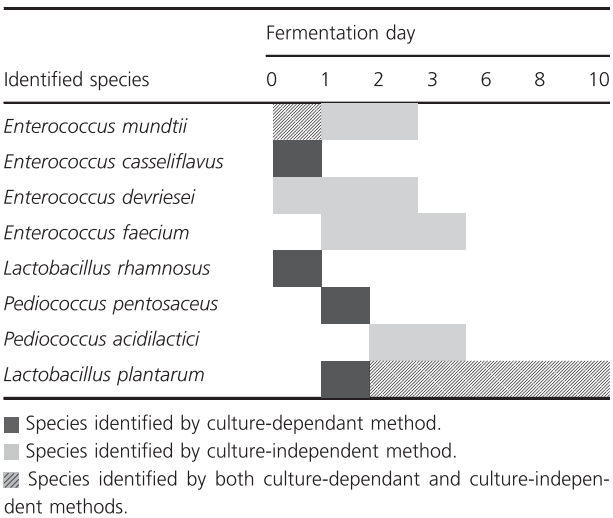


Figure 3 PCR-DGGE profiles of 16S rRNA amplicons from amaranth sourdough samples corresponding to different backslopping times. Sequence characterization of the excised fragments indicated the presence of (1) *Klebsiella oxytoca*; (2) *Enterobacter*; (3) *Enterococcus devriesei*; (4) *Enterococcus faecium*; (5) *Enterococcus muntdii*; (6) *Escherichia blattae*; (7) *Lactobacillus plantarum*; (8) *Pediococcus acidilactici*; (9) *Clostridium* sp.; (10) *Cyanobacterium*; (11 and 12) *Lactobacillus plantarum.*

Table 2 Lactic acid bacteria detected by culture-dependant and culture-independent methods in amaranth sourdoughs



different enterococci species (*Ent. faecium* and *Ent. devriesei*) were identified by PCR-DGGE. *Ent. faecium* is associated with vegetable foods, water and soil (Abriouel *et al.* 2008), while *Ent. devriesei* constitutes an infrequent species in vegetables being associated with meat environments (Martin *et al.* 2009). Enterococci are unable to survive a long-term acidification; however, they play a crucial role at the beginning of fermentation due to their ability to degrade maltose, accumulating glucose, which may be then utilized by maltose-negative strains (Corsetti *et al.* 2007; Weckx *et al.* 2010). Although *Pediococcus* genus was retrieved by both approaches, different species were identified, the presence of *Ped. pentosaceus* and *Ped. acidilactici* being in coincidence with those reported from cereal and noncereal flours and sourdoughs (Corsetti *et al.* 2007; Sterr *et al.* 2009; De Vuyst *et al.* 2014; Ruiz Rodríguez *et al.* 2016). *Lact. rhamnosus* was

detected at day 0 (amaranth flour), its presence being also reported from Italian sourdoughs (De Vuyst *et al.* 2014). Furthermore, *Lact. plantarum* strains were identified by both approaches, persisting up to the end of fermentation. *Lact. plantarum* was also retrieved from spontaneous sourdoughs prepared with amaranth flours (Sterr *et al.* 2009). As recently reported by De Vuyst *et al.* (2014), this species is among the prevailing LAB during sourdough fermentation, regardless of the flour type used, its robustness to survive daily propagation in sourdough environments was investigated (Minervini *et al.* 2010).

Technological-functional and safety characterization of LAB strains

During sourdough fermentation, fast pH decrease is among the most important technological feature of LAB. Selected representative strains from AS showed high acidifying capacities with a rapid pH decrease of sterile amaranth flour extract (SAFE), reaching a pH below 4 after 72 h of fermentation (Table 3). Among the examined functional traits, ropy phenotype was found for *Lact. rhamnosus* CRL1891 and *Ped. pentosaceus* CRL1902. These species are considered EPS-producing strains (Smitinont *et al.* 1999; Péant *et al.* 2005). EPS-producing LAB are particularly promising in GF baking as they can act as hydrocolloids improving volume and texture (Galle *et al.* 2012). *Lact. rhamnosus* CRL1891 was able to hydrolyse amaranth proteins present in SAFE. Although in general sourdough lactobacilli do not possess cell-envelope proteinase activity, a serine proteinase was reported for a human isolate of *Lact. rhamnosus* (Pastar *et al.* 2003). On the other hand, none of the LAB strains were able to degrade starch or produce γ-aminobutiric acid (data not shown).

From a safety point of view, the ability of LAB to inhibit food pathogens and spoilage micro-organisms by the production of antimicrobial compounds is widely known. *Lact. plantarum* CRL1898, *Ent. mundtii* CRL1900 and CRL1953 exhibited anti-*Listeria* activity, while *Lact. plantarum* CRL1898 and *Ped. pentosaceus* CRL1902 were able to inhibit *Bacillus subtilis,* which is responsible for rope spoilage of bread. LAB able to inhibit rope-forming *Bacillus* strains play an important role when used as starters in sourdoughs production (Menteş *et al.* 2007). Moreover, mould growth is the most common spoilage in bakery products leading to huge economic losses and safety reduction due to mycotoxin production. Evaluation of antifungal activity showed that *Lact. rhamnosus* CRL1891,

Table 3 Technological, functional and safety characterization of lactic acid bacteria (LAB) strains from amaranth sourdoughs*

	Acidification†					Antimicrobial activity¶				
	ΔpH	final pH	Hydrolysis of proteins‡	Ropy phenotype§	Production of biogenic amines§	*Listeria*	*Bacillus*	*Aspergillus*	*Penicillium*	ATB resistance**
Enterococcus mundtii CRL1900	2·20	4·15	–	–	+	+	–	–	–	+(Cli)
Enterococcus mundtii CRL1953	2·25	4·10	–	–	–	+	–	–	–	+(Amp/Clin)
Enterococcus casseliflavus CRL1903	2·37	3·98	–	–	+	–	–	+	–	+(Ery/Cli)
Pediococcus pentosaceus CRL1902	2·53	3·82	–	+	+	–	+	–	–	+(Kan/Tet/Str)
Lactobacillus rhamnosus CRL1891	2·59	3·76	+	+	–	–	–	+	+	–
Lactobacillus plantarum CRL1898	2·55	3·80	–	–	–	+	+	+	–	–
Lactobacillus plantarum CRL1956	2·57	3·78	–	–	–	–	–	+	–	–
Lactobacillus plantarum CRL1957	2·54	3·81	–	–	–	–	–	–	–	–

*Values are averages from at least three independent experiments. Standard deviations were <5%.
†pH values after LAB growth in a sterile amaranth flour extract (SAFE); ΔpH: pH (72 h) – pH (0 h); final pH: pH (72 h).
‡Positive (+), indicates protein hydrolysis determined by the OPA method; negative (–), indicates not protein hydrolysis.
§Positive for ropy phenotype or biogenic amines; –, negative for ropy phenotype or biogenic amines.
¶Inhibitory activity was expressed as + (halo presence) or – (no halos) around the spot.
**LAB with MIC higher than the EFSA breakpoints are considered as resistant strains; – indicates susceptible strains according to EFSA breakpoints.
Cli, clindamycin; Amp, ampicillin; Ery, erythromycin; Kan, kanamycin; Str, streptomycin; Tet, tetracycline.

Ent. casseliflavus CRL1903 and *Lact. plantarum* CRL1898 and CRL1956 were able to inhibit mostly *Aspergillus oryzae* , whereas *Lact. rhamnosus* CRL1891 was the only strain capable to inhibit both assayed fungal species. These results are in coincidence with the antifungal activity reported for *Lactobacillus* and *Enterococcus* species of different origins (Schnürer and Magnusson 2005; Oliveira *et al.* 2014). On the other hand, tyrosine-decarboxylase activity was observed for *Ped. pentosaceus* CRL1902, *Ent. mundtii* CRL1900 and *Ent. casseliflavus* CRL1903. These species were reported as tyramine producer LAB involved in vegetable fermentations as wine and cider (Coton *et al.* 2010). As required by EFSA, as part of its Qualified Presumption of Safety (QPS) approach for the safety assessment of bacteria deliberately introduced in the food chain, acquired antimicrobial resistance determinants to antibiotics of clinical importance should be absent. Accordingly, four out of eight LAB strains (*Lact. rhamnosus* CRL1891, *Lact. plantarum* CRL1898, CRL1956 and CRL1957) were susceptible to all examined antibiotics, indicating these strains could be safely used as sourdough starter cultures. As recently reported by Coda *et al.* (2014), nonconventional and GF flours are reservoirs of competitive LAB that can be exploited as starter culture for novel functional foods. In this study, *Lact. plantarum* CRL1898 as a dominant and persisting strain may be selected as a candidate for functional starter culture for amaranth flour fermentation.

Materials and methods

Sourdough preparation and sampling

Commercial amaranth flour (Sturla®, Buenos Aires, Argentina) was used for spontaneous laboratory sourdoughs production. For dough preparation, 100 ml of sterile water were mixed with 100 g of amaranth flour in 500-ml flasks by using a dough kneader (Philips, Buenos Aires, Argentina), with a dough yield [(dough mass/flour mass) × 100] of 200. After incubation of homogeneous mass at 30°C for 24 h, 10 g of ripe sourdough were used to inoculate a fresh water–flour mixture (90 ml of water and 100 g of amaranth flour) in a second sterile flask on day 2. Again, this dough was mixed and incubated under the described conditions. Sourdoughs were daily propagated for 10 days, and samples were taken after 0 (dough), 1, 2, 3, 6, 8 and 10 (sourdough) days. The pH values were determined by using a puncture electrode of a portable pH meter (Sartorius PT-10, Göttingen, Germany). Samples (50 g) were kept at 4°C and analysed within 2 h after collection. A portion of each sample was stored at −20°C for culture-independent microbiological analysis.

LAB community dynamics of backslopped amaranth sourdoughs analysed through a culture-dependent and culture-independent approach

A ten-fold dilution series of sourdough samples were plated on PCA (Britania, Buenos Aires, Argentina) and Man-Rogosa-Sharpe-5 (MRS-5; Meroth *et al.* 2003) supplemented with cycloheximide (0·1 g l^{-1}) and incubated at 30°C for 48 h aerobically and under anaerobiosis (Anaero-Jar; Oxoid, Hampshire, UK) respectively. After counting, 10–20 colonies per sourdough sample were randomly picked from MRS-5 agar plates and were subjected to streaking to purity, Gram staining and catalase reaction. After stored with glycerol at −80°C, isolated strains were deposited at CERELA Culture Collection. Presumptive LAB were subjected to genotypic characterization by RAPD-PCR analysis and identification by sequence analysis of 16S rRNA genes. Strain differentiation was performed by RAPD-PCR analysis using single primers XD9 and M13b as described by Fontana *et al.* (2005a). Resulting amplicons were separated by electrophoresis on 1·5% (w/v) agarose gel and visualized by UV transillumination after staining with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA). The 1 kb DNA ladder (Invitrogen, Buenos Aires, Argentina) was used as molecular size marker. RAPD-PCR profiles were analysed with the Scientific Image Processing (IMAGE J 1.47v) software (National Institutes of Health, Bethesda, MD). Genotypic identification of isolates with different RAPD-PCR profiles was carried out by partial 16S rRNA gene sequencing according to Hebert *et al.* (2000) by using an ABI 3130 DNA sequencer (Applied Biosystems, Foster, CA). Identification queries were fulfilled by a BLAST search (Altschul *et al.* 1990) in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) or in the Ribosomal Database Project (Cole *et al.* 2009). Sequences of the 16S rRNA determined were submitted to GenBank database under the accession numbers shown in Table 1. The main components of the bacterial communities in amaranth sourdoughs as well as their dynamics were investigated through a culture-independent approach. DGGE of amplicons of the V3 region of the 16S rRNA gene was performed by using an INGENY phorU-2 (Ingeny International BV, Goes, the Netherlands) DGGE system followed by purification and sequencing of the DGGE bands as described by Fontana *et al.* (2005b).

Technological, functional and safety characterization

Acid production was assayed in a sterile amaranth flour extract (SAFE) as reported by Alfonzo *et al.* (2013). Ropiness was examined by the presence of a ropy condition after touching the colony with a loop as described by Ruiz Rodríguez *et al.* (2016). For proteolytic activity, LAB

isolates were grown in SAFE broth and the spectrophotometric *o*-phthaldialdehyde test (OPA) procedure (Church *et al.* 1983) was applied. Antibacterial activity of LAB isolates was determined against *Listeria monocytogenes* FBUNT (UNT, Tucumán, Argentina) and *B. subtilis* 168 (PROIMI-CONICET, Tucumán, Argentina) as sensitive strains by the agar spot test (Fontana *et al.* 2015). Plates were incubated at 30°C for 48 h and the presence or absence of inhibition zones around the spots were considered as (+) or (−) results. Antifungal activity against *A. oryzae* and *Penicillium roqueforti* was investigated by a modified agar diffusion assay (Magnusson and Schnürer 2001). Plates were examined for clear zones of inhibition around the LAB streaks and scored as – (no growth suppression) and + (1–5 mm growth suppression). Amino acids decarboxylase activity was tested according to Bover-Cid and Holzapfel (1999). The minimum inhibitory concentrations (MICs) of nine antibiotic (gentamicin, kanamycin, streptomycin, erythromycin, chloramphenicol, tetracycline, ampicillin, clindamycin and vancomycin) were determined for LAB isolates according to ISO 10932:2010 standards. LAB isolates were classified as susceptible or resistant according to the cut-off values proposed by EFSA (2012).

Statistical analysis

Statistical analyses were performed with the software package MINITAB ver. 14 (Minitab Inc, State College, PA) using ANOVA General Linear Models followed by a Tukey's post hoc test, and $P < 0{\cdot}05$ was considered significant. Unless otherwise indicated, all values were the means of three independent trials ± standard deviation. No significant differences were observed between individual replicates.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Microbial species identification after sequencing the variable V3 region of the 16S rRNA gene from DGGE-PCR profiles of amaranth sourdoughs samples.