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ORIGINAL ARTICLE

Biodiversity and technological-functional potential of lactic acid bacteria isolated from spontaneously fermented quinoa sourdoughs

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Abstract

Aims: To analyse lactic acid bacteria (LAB) diversity and technological-functional and safety properties of strains present during spontaneous fermented quinoa sourdoughs.

Methods and Results: Fermentation was performed by daily backslopping at 30°C for 10 days. Autochthonous LAB microbiota was monitored by a biphasic approach combining random amplified polymorphic DNA (RAPD)-PCR and rRNA gene sequencing with PCR-denaturing gradient gel electrophoresis (DGGE) analysis. Identification and intraspecies differentiation allowed to group isolates within nine LAB species belonging to four genera. A succession of LAB species occurred during 10-days backslopping; *Lactobacillus plantarum* and *Lactobacillus brevis* were detected as dominant species in the consortium. The characterization of 15 representative LAB strains was performed based on the acidifying capacity, starch and protein hydrolysis, γ-aminobutyric acid and exopolysaccharides production, antimicrobial activity and antibiotic resistance.

Conclusion: Strains characterization led to the selection of *Lact. plantarum* CRL1905 and *Leuconostoc mesenteroides* CRL1907 as candidates to be assayed as functional starter culture for the gluten-free (GF) quinoa fermented products.

Significance and Impact of the Study: Results on native LAB microbiota present during quinoa sourdough fermentation will allow the selection of strains with appropriate technological properties to be used as a novel functional starter culture for GF-fermented products.

Introduction

Andean regions are centre of origin and domestication of a large number of plant species. Among them, ancestral grain quinoa (*Chenopodium quinoa* Willd) is a crop of the Incas with a high seed yield. Due to its large genetic variability, it is adapted to diverse agro-climatic habitats and edaphic conditions from Colombia to the South of Chile, from sea level (Chilean coast) up to more than 4000 m.a.s.l. This ancestral Andean grain has attracted attention because of the quality and nutritional value of its proteins (15–17%), as an important source of essential amino acids which are limited in traditional cereal flours and the exceptional balance between oil, protein and fat (Vega-Galvez *et al.* 2010; Nascimento *et al.* 2014). In addition, the gluten-free (GF) characteristic of quinoa flour has drawn increased attention for a growing amount of people suffering from celiac disease (Alvarez-Jubete *et al.* 2009). Thus, there is an emergent market for new bakery products manufactured using GF grains. However, as the use of quinoa flour is limited due to the low baking and sensory quality of the final products,

strategies such as fermentation could improve both characteristics (Gallagher *et al.* 2003).

Sourdough is the foremost fermentation used for baking purposes and it has proven ideal for obtaining the appropriate texture, palatability, aroma, shelf life and nutritional value of traditional bread. Thus, the use of sourdough in GF baking may be the new frontier for improving the quality, safety and acceptability of GF baked products (Coda *et al.* 2014). Despite the increasing interest in GF grains and flours, only a few results are available regarding the microbiology of Andean grain sourdoughs and no straightforward conclusions can be made due to the scarce information available (De Vuyst *et al.* 2014). Understanding the microbial composition during fermentation at the species and strain level is particularly important for the correct management of sourdough. The establishment and stability of microbial consortia in sourdoughs depends on the microbial communities and chemical composition of the raw materials as well as the interactions between micro-organisms and fermentation parameters such as temperature, inoculum size, dough yield and time of fermentation (Meroth *et al.* 2003; Vogelmann and Hertel 2011; Ercolini *et al.* 2013). The microbial composition of traditional sourdoughs from various European countries has been investigated in numerous studies (Meroth *et al.* 2003; Weckx *et al.* 2010; Minervini *et al.* 2012; Rocha and Malcata 2012), which revealed great lactic acid bacteria (LAB) diversities. Almost all the features that are attributed to sourdough are mainly consequence of LAB metabolism (De Vuyst and Neysens 2005; Gobbetti *et al.* 2005). For monitoring sourdough-associated LAB species, culture-dependent and -independent methods such as denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA fragments were widely applied (Cocolin *et al.* 2013).

On the other hand, typical features derived from LAB metabolic activities in sourdough will determine the final quality of GF products (Gänzle 2014). In view to design novel functional starter cultures for GF products, it would be necessary the exploitation of sourdough-resident LAB with technological (acidification, proteolytic and amylolytic activity), functional (exopolysaccharides and bioactive compounds production) and safety (antimicrobial activity, antibiotic susceptibility and lack of biogenic amine production) competence (Coda *et al.* 2014). Thus, the aim of this study was to identify predominantly growing LAB during spontaneously fermented quinoa sourdoughs by means of a biphasic approach combining random amplification of polymorphic DNA-PCR (RAPD-PCR) and 16S rRNA gene sequencing and PCR-DGGE analysis. Additionally, technological, functional and safety characterization of LAB isolates was carried out in order to select strains with interesting properties that are relevant for their rational exploitation as functional starter culture for GF quinoa flour fermentation.

Materials and methods

Laboratory sourdough preparation and sampling

Quinoa flours, commercial Yin Yang (Dietética Científica, Buenos Aires, Argentina; QY) and natural Real Hornillos (CAUQUEVA, Jujuy, Argentina; QR), were used in the preparation of spontaneous laboratory sourdoughs. Quinoa flours were obtained from milled quinoa seeds with the following proximate composition (%): moisture, 11·3; protein 12·1; fat, 6·31; ash, 2·01; fibre, 10·4; starch, 57·2 and amylose 19·7 (Nascimento *et al.* 2014). For dough preparation, 100 ml of sterile water were mixed with 100 g of quinoa flour in sterile 500-ml flasks by using a dough kneader (Philips, Buenos Aires, Argentina), with a resulting dough yield [(dough mass/flour mass) × 100] of 200. The homogeneous mass was incubated at 30°C for 24 h. After incubation, 10 g of ripe sourdough were used to inoculate a fresh water–flour mixture (90 ml of water and 100 g of quinoa flour) in a second sterile flask on day 2. Again, this dough was thoroughly mixed and incubated under the conditions described above. Sourdoughs were daily propagated for 10 days, and samples were taken after 0 (dough), 2, 3, 6, 8 and 10 (sourdough) days of fermentation. The pH values were determined by using a puncture electrode of a portable pH-meter (Sartorius PT-10, Gotinga, Germany). Samples (50 g) were cooled down to 4°C and analyzed within 2 h after collection. At each sampling point, a sample portion was stored at −20°C for culture-independent microbiological analysis through PCR-DGGE.

Culture media, enumeration and lactic acid bacteria isolation

Each sample (10 g) was suspended in 90 ml of sterile 0·85% (w/v) saline solution, homogenized for 3 min (Stomacher 400, Seward, Worthing, UK) and serially diluted. Decimal dilutions were plated in triplicates on PCA (Britania, Buenos Aires, Argentina) and Man-Rogosa-Sharpe-5 (MRS-5) agar described by Meroth *et al.* (2003) supplemented with cycloheximide (0·1 g l^{-1}). Total mesophilic bacteria were determined on PCA (Britania) incubated aerobically while putative LAB were incubated under anaerobiosis (AnaeroJar, Oxoid, UK), both at 30°C for 48 h. After counting, means and standard deviations were calculated, and an average of 20–25 colonies per sourdough sample were randomly picked from MRS-5 agar

plates containing
100–300 colonies. Gram-positive and catalase negative bacteria (presumptive LAB) were purified by successive streaking on MRS-5 agar plates, and stored in glycerol at −80°C for further experimentations. The isolated strains were deposited at CERELA Culture Collection.

Molecular identification by culture-dependent methods

Potential LAB isolates 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 in a 25 μl reaction mix using single primers XD9 (5′-GAAG TCGTCC-3′) and M13b (5′-GAGGGTGGCGGTTCT-3′) as previously described by Fontana *et al.* (2005). 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 Plus DNA ladder (Invitrogen, Buenos Aires, Argentina) was used as a molecular size marker. RAPD-PCR profiles were analysed with the Scientific Image Processing ImageJ 1.47v software (National Institutes of Health, Bethesda, MD). LAB isolates were subjected to RAPD-PCR analysis at least twice. Molecular identification of LAB isolates with different RAPD-PCR patterns was carried out by partial 16S rRNA gene sequencing. Genomic DNA was extracted according to Pospiech and Neumann (1995). Oligonucleotide primers (PLB16, 5′-AGAGTTTGATCCTGGCTCAG-3′ and MLB16, 5′-GGCTGCTGGCACGTAGTTAG-3′) were used to amplify the variable V1 region of the 16S ribosomal RNA gene according to Hebert *et al.* (2000). PCR products were electrophoresed in 1% (w/v) agarose gels, stained and visualized as described above. Amplicons were excised from the gel, purified using a GFX PCR DNA gel band purification kit (GE Healthcare, Amersham, UK), and sequenced at CERELA-CONICET by using an ABI 3130 DNA sequencer (Applied Biosystems, Foster, CA, USA). rRNA gene sequence alignments were performed using the multiple sequence alignment method and identification queries were fulfilled by a BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) and in the Ribosomal Database Project (Cole *et al.* 2009). Determined 16S rRNA sequences were submitted to the GenBank database.

Molecular identification by culture-independent methods

To investigate the dominant bacterial community dynamics of quinoa sourdoughs, PCR-DGGE analysis was applied. FastDNA SPIN kit and the FastPrep instrument (MP Biomedicals, Santa Ana, CA, USA) were used for the extraction of DNA directly from 200 mg of each ripe quinoa sourdough sample according to manufacturer's recommendations. DNA obtained directly from sourdough corresponding to 0, 1, 2, 3, 5, 8 and 10 refreshment steps were amplified by PCR. Primers V3f (GC), 5′-CCGGGGGGCGCGCCCCGGGCGGGGCGGGGACGGG-GGGCCTACGGGAGGCAGCAG-3′) and Uni-0515r (5′-A TCGTATTACCGCGGCTGCTGCTGGCA-3′) were used to amplify the V3 region of bacterial 16S rRNA gene according to Fontana *et al.* (2005). PCR amplifications were performed with the PCR Master Mix (Promega, Milan, Italy) in a GeneAmp PCR System 9700 (Applied Biosystems) programmed following an initial DNA denaturation for 5 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 58°C, 1 min at 72°C and a final extension of incomplete products for 7 min at 72°C. PCR-DGGE was performed using an INGENY phorU-2 (Ingeny International BV, Goes, Netherlands) DGGE system. PCR products were run on an 8% (w/v) acrylamide gel with a 40–60% linear gradient of urea and formamide and electrophoresed at 90 V, 60°C for 16 h in 1× TAE (40 mmol l^{-1} Tris-Acetate, 2 mmol l^{-1} EDTA, pH 8) buffer. Denaturing gradient gels were stained with 1× SYBR Green I (Roche, Monza, Italy) for 15 min and photographed under UV illumination. DGGE bands were excised from the gel, placed in 50 μl of nuclease-free water and stored overnight at 4°C to elute DNA. Eluted DNA was re-amplified as previously described. To make sure that no biases were introduced by the PCR re-amplification, the selected bands were subjected to DGGE analysis before sequencing. A co-migration between the original band cut from the DGGE gel and the re-amplified band was always obtained, thereby confirming the validity of the used approach. PCR products of correct mobility were then amplified with the primer without GC clamp, purified and directly sequenced (BMR Genomics, Padova, Italy).

Technological-functional and safety properties of LAB isolates

Acidification activity

The kinetics of acidification by LAB isolates was evaluated in a sterile flour extract (SFE) liquid broth as described by (Alfonzo *et al.* 2013). Briefly, 160 g of commercial quinoa flour (Yin Yang) were suspended in 750 m1 of distilled H_2O, sterilized at 121°C for 20 min, precipitated and the supernatant was used as liquid media. LAB isolates were grown in MRS broth (Biokar Diagnostics, Allonne, France) at 30°C for 16 h. To eliminate carry-over nutrients, cells were harvested by centrifugation (7000 ***g***, 7 min), washed twice with 0·85% (w/v) sterile saline and re-suspended to a final OD_{560} of 0·5 in the same solution. Cell suspensions were used to inocu-

late (2%) the SFE that was incubated at 30°C for 72 h. Samples for pH measurements were withdrawn at 0, 4, 8, 24, 48 and 72 h after inoculation.

Proteolytic activity
LAB isolates were grown in SFE broth as described above and proteolytic activity was evaluated spectrophotometrically using the *o*-phthaldialdehyde test (OPA) procedure of Church *et al.* (1983), by measuring the increase in absorbance at 340 nm relative to uninoculated SFE broth.

Amylolytic activity
Starch degradation was investigated by spot inoculation of active LAB isolates (5 μl) on plates containing MRS-starch medium in which glucose was replaced by starch (1%). Inoculated plates were allowed to grow at 30°C for 48 h and then stored at 4°C during 24 h before being flooded with iodine solution (4%). Amylase production was indicated by a clear zone around the colonies, while the rest of the plate stained blue-black. *Lactobacillus amylophilus* CECT4133 was used as positive control strain.

Ropy phenotype
Active cultures of LAB strains were spot inoculated on MRS agar and incubated at 30°C during 2–7 days. Ropiness was examined by the presence of a ropy condition after touching the colony with a loop.

γ-aminobutyric acid production
γ-Aminobutyric acid (GABA) production was preliminary screened by the inoculation of LAB isolates in MRS medium containing monosodium glutamate (5%) (MRSG) and incubated at 30°C during 72 h. The pH was measured at 24, 48 and 72 h and those strains exhibiting an increase in the pH at 72 h were considered as presumptive GABA producers. GABase assay (Sigma-Aldrich, St. Louis, MO, USA) was used to determine GABA concentration as reported by Tsukatani *et al.* (2005). Briefly, 10 μl of each strain supernatant was incubated for 10 min at 37°C with the assay mixture containing 80 mmol l^{-1} Tris buffer (pH 9), 750 mmol l^{-1} sodium sulphate, 10 mmol l^{-1} dithiothreitol, 1·4 mmol l^{-1} $NADP^+$, 2 mmol l^{-1} α-ketoglutarate and 0·3 g l^{-1} GABase in each well of a 96-well microtitre plate and the optical density at 340 nm was measured every 30 s. GABA concentration was calculated by using a calibration curves generated with standard solutions containing 0, 1, 2, up to 10 mmol l^{-1} GABA prepared in the same sterile medium used for cultures growth.

Antimicrobial activity
Antibacterial activity of LAB isolates was determined against *Listeria monocytogenes* FBUNT (Facultad de Bioquímica, Química y Farmacia, UNT, Argentina) and *Bacillus subtilis* 168 (PROIMI-CONICET) as sensitive strains by the agar spot test (Fontana *et al.* 2015). Overnight LAB cultures were centrifuged (7000 ***g***, 15 min), and the supernatants adjusted to pH 6·5. Supernatants (5 μl) were spotted onto 7 ml of BHI agar plates (0·7% w/v) previously inoculated with 50 μl of each indicator strain. Plates were incubated at 30°C for 48 h and the presence of a clear inhibition zone around the spots was considered as a positive antagonistic effect. Inhibitory activity was expressed as + (halo presence) or − (no halos) around the spot. Antifungal activity against *Aspergillus oryzae* and *Penicillium roqueforti* was investigated by a modified agar diffusion assay (Magnusson and Schnürer 2001). Petri plates containing Sabureaud agar (Britania) were inoculated with the fungus and incubated at 25°C during 48 h. After mycelial colonies development, spores were collected and adjusted to 10^5 per ml of sterile saline solution (0·85% w/v). LAB strains were streak inoculated on MRS agar plates and after incubation at 30°C for 48 h; plates were overlaid with 10 ml of Sabureaud soft agar (0·7% agar) containing fungal spores suspensions (10^4 per ml) and incubated aerobically at 30°C for 48 h. Then, plates were examined for clear zones of inhibition around the LAB streaks and scored as – (no growth suppression) and + (1–5 mm growth suppression).

Biogenic amines production
The ability to decarboxylate amino acids used as precursor was tested according to Bover-Cid and Holzapfel (1999). Briefly, the plates with the agar medium, supplemented with histidine and tyrosine (20 mg l^{-1}) were spotted with the active LAB strain and incubated anaerobically at 30°C for 2–5 days. Growth of decarboxylating strains was easily recognizable because of their purple halo in the yellow medium.

Antibiotic susceptibility
The antibiotics recommended for the European Food Safety Authority (EFSA, 2012) to identify bacterial strains with potential acquired resistance to antibiotics were analysed. The antibiotics tested were: ampicillin (Amp; 0·032–16 μg ml^{-1}), vancomycin (Van; 0·25–128 μg ml^{-1}), chloramphenicol (Chl; 0·125–64 μg ml^{-1}), gentamycin (Gen; 0·5–256 μg ml^{-1}), streptomycin (Str; 0·5–256 μg ml^{-1}), kanamycin (Kan; 2–1024 μg ml^{-1}), tetracycline (Tet; 0·125–64 μg ml^{-1}), erythromycin (Ery; 0·016–8 μg ml^{-1}) and clindamycin (Clin; 0·032–16 μg ml^{-1}). The minimum inhibitory concentration of antibiotics (MIC) was determined by the broth microdilution method reported by the ISO 10932/IDF 233 standard (ISO, 2010). The strains were classified as susceptible or resistant according to the cut-off values proposed by EFSA (2012). A bacterial strain was defined as

susceptible when it is inhibited at a specific antimicrobial concentration equal or lower than the established cut-off value and it is considered as resistant when it is not inhibited at a concentration higher than the established cut-off value.

Statistical analysis

Statistical analyses were performed with the software package MINITAB 14 (Minitab Inc., State College, PA, USA) 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.

Results

Microbiological analyses

In this study, quinoa flours (Yin Yang, QY and Real Hornillos QR) were used to prepare spontaneous laboratory sourdoughs. The course of total mesophiles and LAB counts in PCA and MRS-5, respectively, as well as the pH during QR and QY sourdough fermentation are shown in Table 1. Fermentation was characterized by a rapid increase in LAB number right from the beginning of fermentation, the major changes occurring within the first 3 days of propagation. LAB counts increased from 1·65 and 2·60 log CFU g^{-1} to a final value of 8·43 and 8·15 log CFU g^{-1} on tenth day for QR and QY sourdoughs, respectively (Table 1), while the total bacterial count during 10 days of backslopping varied from 3·0–3·8 to 8·3–8·6 log CFU g^{-1} for both sourdoughs. The trend for the total mesophiles paralleled those of the LAB population throughout fermentation. Thus, the preponderance of LAB during QR and QY fermentation was in correlation with final pH values, which decreased from *c.* 6·4 and 6·0 at the beginning of the fermentation to 4·2 and 3·9 at day 3 for QR and QY doughs respectively, both sourdoughs reaching a final pH of *c.* 3·8 (Table 1).

Isolation, identification and dynamics of dominant LAB

Based on Gram staining and catalase test results, 97 isolates (37 cocci and 60 rods) from QR and 68 isolates (10 cocci and 58 rods) from QY samples were selected as presumptive LAB. All isolates (165) were subjected to RAPD-PCR analysis by using the primers M13b and XD9. Strains showing identical RAPD band patterns were considered as one RAPD-biotype (Bt); isolates were grouped as belonging to 12 M13b and 14 XD9 different RAPD-biotypes (Fig. 1). At least one representative from each RAPD-biotype was identified by partial 16S rRNA gene sequencing; these strains were deposited at CERELA Culture Collection and a CRL number was assigned. In Table S1, biotype information for RAPD-PCR obtained with M13b and XD9 oligonucleotides for LAB isolates is reported. M13b RAPD-biotypes (M13b-Bt) of QR sourdough isolates (Fig. 1a; Table S1) were associated with *Enterococcus hermanniensis* (Bt1 and Bt2), *Enterococcus casseliflavus* (Bt3), *Enterococcus mundtii* (Bt4), *Lactococcus lactis* (Bt5), *Leuconostoc citreum* (Bt7) and *Lactobacillus plantarum* (Bt12) as well as *Lactobacillus brevis* (Bt9, Bt10 and Bt11). RAPD-biotypes from QY isolates were associated with *Leuconostoc mesenteroides* (Bt6) and *Lact. plantarum* (Bt8). On the other hand, when primer XD9 was used (Fig. 1b; Table S1), QR biotypes were associated with *Ent. hermanniensis* (Bt1 and Bt2), *Ent. casseliflavus* (Bt3), *Ent. mundtii* (Bt4), *Leuc. citreum* (Bt6), *Leuc. mesenteroides* (Bt7) and *Lact. plantarum* (Bt14) as those found with M13b primer as well as *Lact. brevis* (Bt9, Bt10, Bt11 and Bt13), while QY isolates were associated with *Lact. plantarum* (Bt8) and *Lact. brevis* (Bt12). By applying primer XD9, it was unable to distinguish between *Lc. lactis* and *Leuc. mesenteroides* isolated from QR and QY sourdoughs, respectively, since the same band pattern (Bt5) was obtained.

Table 1 Viable cell-counts of total bacteria (PCA agar) and lactic acid bacteria (LAB) (MRS-5 agar) during spontaneous fermentation of quinoa Real Hornillos (QR) and quinoa Yin Yang (QR) sourdoughs for 10 days*

	QR sourdough			QY sourdough		
Days	LAB counts (log CFU g^{-1})	Total bacteria counts (log CFU g^{-1})	pH	LAB counts (log CFU g^{-1})	Total bacteria counts (log CFU g^{-1})	pH
0	1·65 ± 0·2	3·74 ± 0·3	6·4 ± 0·2	2·60 ± 0·2	3·01 ± 0·2	6·0 ± 0·2
1	6·40 ± 0·3	6·70 ± 0·3	4·9 ± 0·1	5·34 ± 0·3	6·62 ± 0·2	5·7 ± 0·1
3	7·52 ± 0·4	7·43 ± 0·3	4·2 ± 0·2	7·41 ± 0·3	7·76 ± 0·3	3·9 ± 0·2
6	8·40 ± 0·4	8·38 ± 0·4	3·9 ± 0·2	7·78 ± 0·4	7·90 ± 0·3	3·8 ± 0·1
8	8·32 ± 0·4	8·42 ± 0·4	3·9 ± 0·1	8·11 ± 0·3	8·08 ± 0·4	3·8 ± 0·2
10	8·43 ± 0·4	8·57 ± 0·4	3·9 ± 0·2	8·15 ± 0·3	8·28 ± 0·3	3·8 ± 0·2

*Values are the means ± standard deviations from three independent experiments.

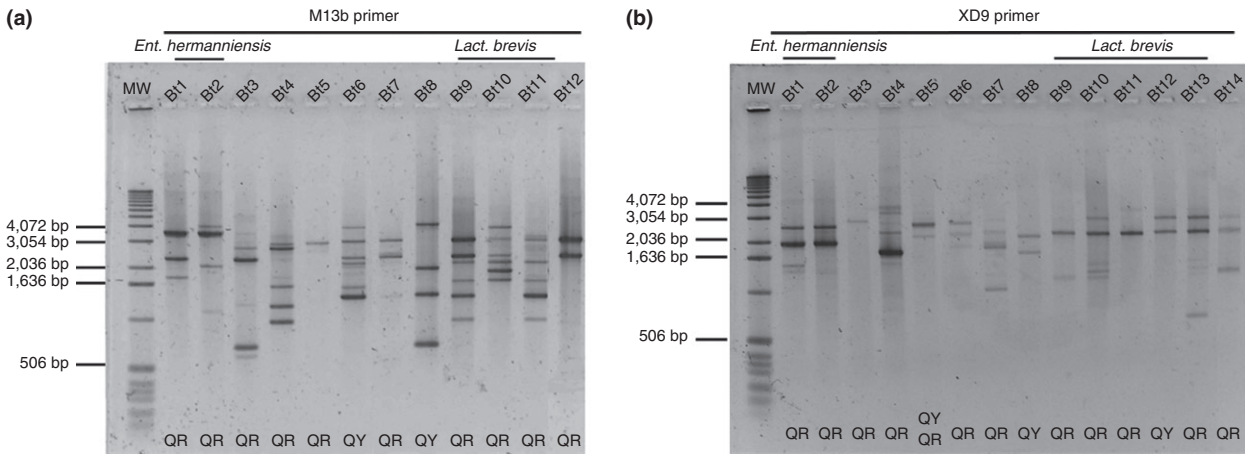

Figure 1 Representative biotypes random amplified polymorphic DNA (RAPD)-PCR profiles of lactic acid bacteria (LAB) isolated during quinoa Real Hornillos (QR) and quinoa Yin Yang (QY) sourdoughs fermentation. Primers M13b (a) and XD9 (b) were used for RAPD-PCR analysis. M, Molecular weight marker (1 kb DNA ladder, Invitrogen). (a) M13b RAPD profiles including the following biotypes: M13b-Bt1 (*Enterococcus hermanniensis*, QR); M13b-Bt2 (*Ent. hermanniensis*, QR); M13b-Bt3 (*Enterococcus casseliflavus*, QR); M13b-Bt4 (*Enterococcus mundtii*, QR); M13b-Bt5 (*Lactococcus lactis*, QR); M13b-Bt6 (*Leuconostoc mesenteroides*, QY); M13b-Bt7 (*Leuconostoc citreum*, QR); M13b-Bt8 (*Lactobacillus plantarum*, QY); M13b-Bt9 (*Lactobacillus brevis*, QR); M13b-Bt10 (*Lact. brevis*, QR); M13b-Bt11 (*Lact. brevis*, QR) and M13b-Bt12 (*Lact. plantarum*, QR and QY). (b) XD9 RAPD profiles including XD9-Bt1 (*Ent. hermanniensis*, QR); XD9-Bt2 (*Ent. hermanniensis*, QR); XD9-Bt3 (*Ent. casseliflavus*, QR); XD9-Bt4 (*Ent. mundtii*, QR); XD9-Bt5 (*Lc. lactis/Leuc. mesenteroides*, QR); XD9-Bt6 (*Leuc. citreum*, QR); XD9-Bt7 (*Leuc. mesenteroides*, QR); XD9-Bt8 (*Lact. plantarum*, QY); XD9-Bt9 (*Lact. brevis*, QR); XD9-Bt10 (*Lact. brevis*, QR); XD9-Bt11 (*Lact. brevis*, QR); XD9-Bt12 (*Lact. brevis*, QY); XD9-Bt13 (*Lact. brevis*, QR) and XD9-Bt14 (*Lact. plantarum*, QR and QY).

Species composition and dynamics of LAB population, determined by culture-dependent techniques at each backslopping step for QR and QY sourdoughs are summarized in Table 2. Isolates recovered from quinoa flours (day 0) belonged to the species *Lact. plantarum* for QY samples while a more complex microbiota dominated by cocci such as *Ent. casseliflavus*, *Ent. mundtii*, *Enterococcus hermanniensis* and *Lc. lactis*, was initially present in QR dough. After the first refreshment step (day 1), *Lc. lactis* persisted in QR dough together with *Leuc. mesenteroides* and *Leuc. citreum* while *Lact. plantarum* and *Leuc. mesenteroides* were identified from QY dough. After 6 days of fermentation, a natural selection was produced with the dominance of *Lact. plantarum* and *Lact. brevis* in both sourdoughs. Five and three different biotypes from XD9 and M13b primers respectively, were able to be distinguished (Fig. 1, Table S1), indicating *Lact. brevis* intraspecies diversity.

Table 2 Lactic acid bacteria (LAB) population dynamics during laboratory sourdough fermentations

Quinoa flour*/identified micro-organisms	Backslopping day					
	0	1	3	6	8	10
QR						
Enterococcus caseliflavus	■					
Enterococcus hermaniensis	■					
Enterococcus mundtii	■					
Lactococcus lactis	■	■				
Leuconostoc citreum	■	■				
Leuconostoc mesenteroides	■	■	■			
Lactobacillus brevis	■	■	■	■	■	■
Lactobacillus plantarum	■	■	■	■	■	■
QY						
Leuconostoc mesenteroides		■				
Lactobacillus brevis					■	■
Lactobacillus plantarum	■	■	■	■	■	■

*QR, natural Real Hornillos quinoa; QY, commercial Yin Yang quinoa. Shades indicate the presence of LAB species.

PCR-DGGE fingerprinting of the dominant LAB population from quinoa sourdoughs

Besides traditional culture-dependent microbiological analysis, PCR-DGGE coupled with partial 16S rRNA gene sequencing was performed to follow the bacterial community dynamics in the sourdough ecosystems (Fig. 2). The PCR-DGGE band patterns showed major changes in bacterial community at the beginning of fermentation (0–1 days) and only minor changes occurred subsequently. Fifteen bands were identified by sequencing (Table S2). Bands 1, 2, 6 and 7 from QY sourdough (0–2 days) were identified as belonging to *Enterobacteriaceae*, namely *Klebsiella oxytoca*, *Pantoea ananatis* and *Pantoea* sp., whereas bands 10 and 11 from QR sample (day 0) were identified as *Kl. oxytoca/Salmonella enterica* and uncultured *Enterobacteriaceae*. With the exception of

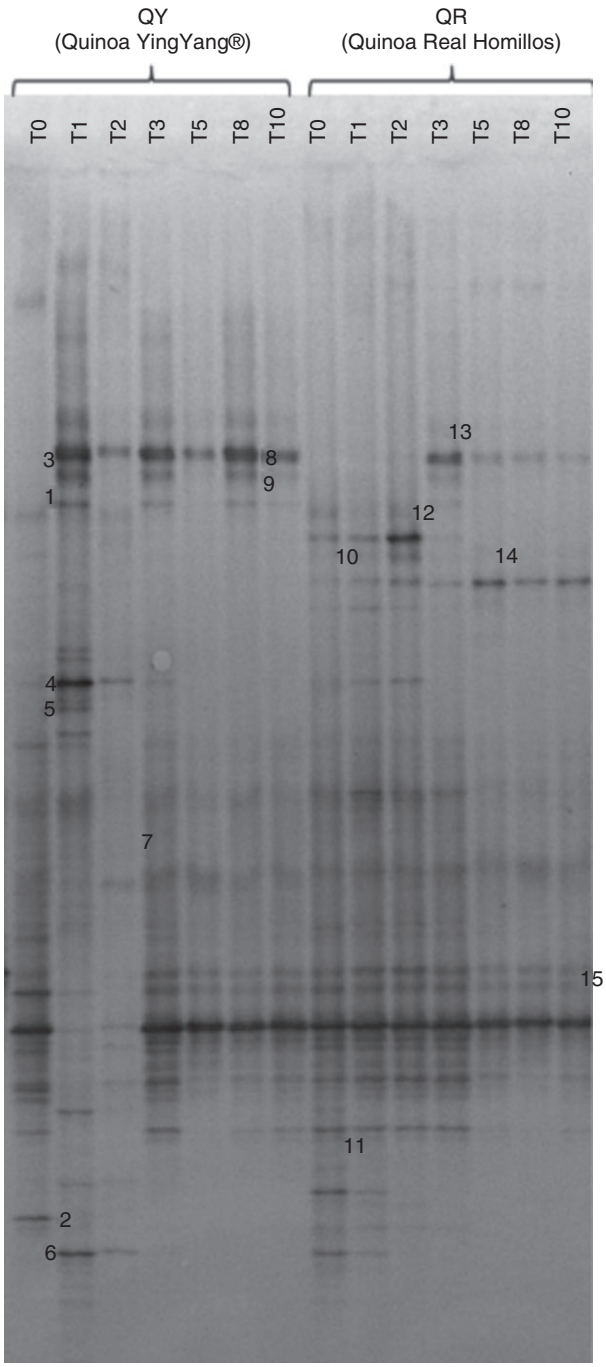

Figure 2 PCR-denaturing gradient gel electrophoresis (DGGE) profiles of 16S rDNA amplicons from quinoa Real Hornillos (QR) and quinoa Yin Yang (QY) sourdough samples corresponding to different back-slopping times. Sequence characterization of the excised fragments indicated the presence of the following: 1 and 10, *Klebsiella oxytoca*/*Salmonella enterica*; 2, *Pantoea ananatis*; 3, 8, 9 and 13, *Lactobacillus plantarum*; 4, *Enterococcus faecium*; 5, *Enterococcus mundtii*; 6 and 7 *Pantoea* sp./*Enterobacter cowani*; 11, uncultured *Enterobacteriaceae*; 12, *Enterococcus devriesei*; 14, *Lactobacillus brevis* and 15, uncultured bacteria/*Lactuca sativa*.

band 7, all these signals were only visible up to day 2 of fermentation. In addition, an intense band (15) from vegetable DNA, identified as *Lactuca sativa* (Phylum Cyanobacteria, Chloroplast) was observed in both sourdoughs samples. All these non-LAB organisms were present in QR and QY flours, since their bands were visualized between 0 and 2 days of fermentation. On the other hand, *Lact. plantarum* was identified (bands 3, 8 and 9) as a dominating LAB species from QY sourdough since it was present from day 1 to 10 while a band (13) corresponding to this species was detected at 3 days of fermentation in QR dough, decreasing its intensity thereafter. Also for QR samples, a faint band (14) increasing its intensity from day 1 to day 10 was identified as *Lact. brevis*, while no amplicons for this species were evidenced during QY fermentation. In addition, from QY samples *Enterococcus faecium* (band 4) and *Ent. mundtii* (band 5) were detected from day 1 to 3 and at day 1, respectively; during QR dough fermentation, a weak band (12) that increased its intensity from day 0 to day 2, was identified as *Enterococcus devriesei*.

Technological, functional and safety characterization of LAB isolates

The acidification kinetics of SFE by LAB strains showed that all strains were able to acidify the medium below pH 4·0 after 72 h with the exception of *Ent. mundtii* (Table 3). *Lactococcus lactis* was the only strain that exhibited proteolytic activity, showing an amino acid released of 1·83 μg ml^{-1} from the SFE extract (data not shown). GABA production screening revealed that all *Lact. plantarum* and *Lact. brevis* strains were able to synthesize this amino acid, *Lact. brevis* CRL1959 being the greatest GABA-producer strain (Table 3). On the other hand, hydrolysis of starch was only displayed by *Ent. mundtii* CRL1896, whereas ropy phenotype was observed for *Leuc. citreum* CRL1904, *Leuc. mesenteroides* CRL1907, *Lact. brevis* CRL1959 and *Lact. plantarum* CRL1905. The amino acid decarboxylase activity assay showed that *Lact. brevis* CRL1951 and CRL1960 produced histamine.

As bread and other baked products can become contaminated with spoilage bacteria or moulds, antimicrobial activity was also assayed (Table 3). Two strains, *Ent. mundtii* CRL1896 and *Ent. casseliflavus* CRL1899 from QR flour, exhibited anti-*Listeria* activity, while *Ent. mundtii* CRL1896, *Leuc. citreum* CRL1904, *Leuc. mesenteroides* CRL1907, *Lact. plantarum* CRL1905 and *Lact. plantarum* CRL1906 (from QR sourdough) were inhibitory against *Bacillus*. The antifungal assays showed that 73% and 33% of the LAB strains were able to inhibit *A. oryzae* and *P. roqueforti* respectively. Several LAB

Table 3 Technological, functional and safety properties of lactic acid bacteria (LAB) isolated from quinoa sourdoughs*

	pH†					Production of		Antimicrobial activity**				
	6 h	24 h	72 h	Hydrolysis of Starch‡	Ropy phenotype¶	γ-Aminobutyric acid (mmol l^{-1})	Biogenic amines¶	*Listeria*	*Bacillus*	*Aspergillus*	*Penicilium*	Antibiotic resistance††
Enterococcus hermanniensis CRL1894	4·44	3·96	3·92	–	–	n.d.§	–	–	–	–	–	Cli
Enterococcus hermanniensis CRL1958	4·21	3·71	3·50	–	–	n.d.	–	–	–	–	–	Tet
Enterococcus casseliflavus CRL1899	5·10	4·40	3·87	–	–	n.d.	–	+	–	+	+	Str/Gen/Cli
Enterococcus mundtii CRL1896	5·50	4·85	4·48	+	–	n.d.	–	+	+	+	–	Ery/Str/Gen/Cli
Lactococcus lactis CRL1895	4·36	3·97	3·87	–	–	n.d.	–	–	–	+	–	–
Leuconostoc mesenteroides CRL1901	4·41	3·99	3·92	–	–	n.d.	–	–	–	+	–	Chl/Kan
Leuconostoc mesenteroides CRL1907	4·17	3·60	3·40	–	+	n.d.	–	–	+	+	+	–
Leuconostoc citreum CRL1904	4·12	3·69	3·55	–	+	n.d.	+	–	+	+	+	Amp
Lactobacillus plantarum CRL1906	4·16	3·70	3·48	–	–	29·13	–	–	+	+	–	–
Lactobacillus plantarum CRL1905	4·16	3·64	3·34	–	+	19·45	–	–	+	+	–	–
Lactobacillus brevis CRL1959	4·29	3·83	3·61	–	+	241·91	–	–	–	+	+	Amp
Lactobacillus brevis CRL1960	5·12	4·22	3·74	–	–	13·04	+ (His)	–	–	–	–	Amp
Lactobacillus brevis CRL1951	5·17	4·17	3·57	–	–	48·84	+ (His)	–	–	–	–	Amp
Lactobacillus brevis CRL1961	4·06	3·66	3·40	–	–	51·71	–	–	–	+	–	Amp
Lactobacillus brevis CRL1962	4·10	3·52	3·32	–	–	28·66.	–	–	–	+	+	Amp

*Values are averages from at least three independent experiments. Standard deviations were <5%.

†pH values after LAB growth in a sterile quinoa flour extract (SFE). The initial pH of the SFE was ~5·96.

‡+, indicates starch hydrolysis on plates containing MRS-starch medium; –, indicates not starch hydrolysis.

§n.d. indicates no detection of GABA production.

¶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.

strains showed antifungal activity against both fungal indicators (Table 3).

Microbiological breakpoints for Amp, Van, Gen, Kan, Str, Ery, Clin, Tet and Chl reported by EFSA on the assessment of bacterial products used as feed additives in relation to antibiotic resistance were used to categorize the isolated LAB as susceptible or resistant strains (EFSA, 2012; Table 3). Particularly for vancomycin, it is known that *Leuconostoc* spp. and facultative and obligate heterofermentative *Lactobacillus* (*Lact. plantarum* and *Lact. brevis*) are intrinsically resistant; therefore vancomycin resistance analysis is not required by EFSA in these bacteria (EFSA, 2012). Four strains (*Lc. lactis* CRL1895, *Leuc. mesenteroides* CRL1907, *Lact. plantarum* CRL1906 and *Lact. plantarum* CRL1905) out of 15 (27%) were susceptible to all assayed antibiotics. Clindamycin and tetracycline resistance was found in *Ent. hermanniensis* CRL1894 and CRL1958 respectively. All *Lact. brevis* strains were resistant to Amp. *Lact. brevis* (CRL1959 and CRL1962) and *Leuc. mesenteroides* CRL1901 displayed a double resistance pattern (Amp/Cli and Chl/Kan respectively). Multiple antibiotic resistance was only detected for *Ent. casseliflavus* CRL1899 (Str/Gen/Cli) and *Ent. mundtii* CRL1896 (Ery/Str/Gen/Cli) respectively.

Discussion

In spite of the increasing interest on GF quinoa flour, there is a lack of information on the LAB microbiota during its fermentation (De Vuyst *et al.* 2014). During quinoa sourdough fermentation, LAB showed to be the dominant microbial population, these results being similar to those previously reported for cereal and GF-cereal flours and sourdoughs (Van der Meulen *et al.* 2007; Weckx *et al.* 2010; De Vuyst *et al.* 2014).

At each backslopping stage, samples were analysed using culture-dependent and culture-independent techniques. This combined approach allowed an optimal survey of LAB microbiota within complex food matrices with unfavourable conditions for bacterial growth, such as the low water content of flour, in which LAB are usually present in a dormant state (Corsetti and Settanni 2007; Alfonzo *et al.* 2013). During the first refreshment steps, LAB cocci species were mainly retrieved. *Enterococcus* and *Lactococcus* have been identified in this study as subdominant sourdough LAB, highlighting the limited importance of these genera in mature sourdoughs where lactobacilli predominate (Corsetti and Settanni 2007; Van der Meulen *et al.* 2007; Weckx *et al.* 2010; De Vuyst *et al.* 2014). *Enterococcus* species represent contaminants from the environment and grains processing. In fact, they have been reported to be present in water, soils, cereal flours and other vegetable materials (De Vuyst and Neysens 2005; Van der Meulen *et al.* 2007). During the first days of fermentation and by using a culture-dependent approach, *Ent. casseliflavus, Ent. mundtii* and *Ent. hermanniensis* were identified from QR sourdough while no species from this genus were detected in QY sourdough. It was shown that quality status of the flour determines the community dynamics and metabolite kinetics of sourdough fermentation processes (De Vuyst *et al.* 2014). Since QY is a commercial brand, it might contain less bacterial load. *Lactococcus lactis* was isolated from QR samples during the first 1–2 days, which has also been associated with cereal kernels and flours during the early stages of wheat and spelt fermentation (De Vuyst and Neysens 2005; Van der Meulen *et al.* 2007; Weckx *et al.* 2010; Ercolini *et al.* 2013). On the other hand, *Leuc. mesenteroides* has been identified from QR and QY sourdoughs, whereas *Leuc. citreum* was only present during QY fermentation, however their counts decreased below the detection limit after the third refreshment step. *Leuconostoc* have been found as secondary population from Belgian and Italian wheat sourdoughs (Weckx *et al.* 2010; Minervini *et al.* 2012) and it showed to be sensitive to acid stress (McDonald *et al.* 1990) decreasing upon further acidification of the sourdough ecosystem.

Although about 30 LAB species are considered typical of sourdough environment, species belonging to *Lactobacillus* genus are the most important group of fermenting bacteria being responsible for the stability over consecutive refreshments of sourdoughs. *Lactobacillus sanfranciscensis*, *Lact. plantarum* and *Lact. brevis* are the LAB species most frequently found (De Vuyst and Neysens 2005; Corsetti and Settanni 2007; Bessmeltseva *et al.* 2014). In this study, *Lact. plantarum* and *Lact. brevis* consortium has been found to dominate laboratory quinoa sourdoughs from day 3 up to the end of fermentation. The prevalence of *Lact. plantarum* during daily propagation of sourdoughs is attributed to its versatile metabolism, the ability to adapt to different environmental conditions and its large antimicrobial spectrum (Minervini *et al.* 2010). On the other hand, *Lact. brevis* was found to predominate in wheat and maize/rye sourdough ecosystems (Minervini *et al.* 2012; Rocha and Malcata 2012). On the basis of a survey of several publications, *Lact. plantarum, Lact. brevis, Lactobacillus fermentum* and *Lact. sanfranciscensis* are the prevailing LAB species during wheat and rye sourdough fermentation (De Vuyst *et al.* 2014). Even though isolation and characterization of LAB from sourdough matrices throughout the world have been reported, no straightforward conclusions about the microbial diversity in buckwheat, amaranth and quinoa can be made due to the low number of publications available (De Vuyst *et al.* 2014).

Biodiversity data obtained by culturing were complementary to PCR-DGGE community fingerprinting. Sev-

eral LAB species identified by culturing were not detected by DGGE and viceversa such as *Lactococcus, Leuconostoc* and *Enterococcus. Enterococcus devriesei* from QR and *Ent. mundtii* and *Ent. faecium* from QY sourdoughs were only detected by PCR-DGGE. This discordance is foreseeable; the inherent biases of DGGE technique and the inability to use a culture medium to support the equal growth of each LAB species, might limit the visualizing of minor bacterial population (Vera *et al.* 2009; Cocolin *et al.* 2013). However, *Lact. plantarum* was detected by both molecular approaches from day 1 to 10 in both sourdoughs. Altogether, a succession of LAB species and a persistence of *Lactobacillus* during quinoa sourdough fermentation were found, confirming that the combination of molecular tools represents the best strategy for detection of microbial communities from complex matrix (Alfonzo *et al.* 2013). Besides, intra-species diversity of *Lact. brevis* and *Lact. plantarum* was only evidenced by RAPD-PCR approach.

In order to select LAB strains for quinoa sourdough starter culture, 15 representative strains from different RAPD-biotypes were investigated for their technological, functional and safety relevant characteristics. During sourdough fermentation, fast pH decrease is among the most important technological feature of LAB. *Lactobacillus* and *Leuconostoc* species showed high-acidifying capacities with a rapid pH decrease in quinoa flour extract. Amylolytic activity was only displayed by *Ent. mundtii* CRL1896 that is in accordance with the general ability of plant-associated enterococci to ferment maltose and in some cases to degrade starch (Shibata *et al.* 2007; Mokhtari *et al.* 2013). To our knowledge, this is the first report for *Ent. mundtii* as amylolytic strain. Metabolism of sourdough microbiota and cereal enzymes are interdependent; acidification modulate the activity of cereal enzymes and in turn, cereal enzymes provide substrates for bacterial growth (Gänzle 2014). Proteolysis evaluation resulted in scarce quinoa protein degradation. *Lactococcus lactis* CRL1895 was the only strain that showed proteinase activity which is in correlation with the presence of a cell-bound extracellular proteinase that adapted to survive in fermented plant materials (Siezen *et al.* 2008). Due to the absence of extracellular proteinase in most sourdough lactobacilli, primary proteolysis depends on quinoa albumins and globulins degradation by grain endogenous proteases (Gänzle 2014).

Glutamate decarboxylase (GAD), which catalyses the conversion of L-glutamate onto the bioactive metabolite GABA, was present in the selected *Lact. plantarum* and *Lact. brevis* strains. Physiologically, the expression of GAD genes is assumed to control cell acidification by decarboxylation of glutamate into the neutral product GABA; this compound after being exported contributes to alkalization of the extracellular medium (Cotter and Hill 2003). *Lact. brevis* was also reported to produce GABA during fermentation of black raspberry juice (Kim *et al.* 2009). GABA-enriched breads should be considered as a promising option for enhancing nutritional, functional, sensory and technological properties of bread (Coda *et al.* 2010). Moreover, the ropy phenotype display by *Leuconostoc* and *Lactobacillus* species in this study is in agreement with those found from similar species during fermentation of rice-based doughs and oat-based medium (Mårtensson *et al.* 2003; Torino *et al.* 2015). The presence of a LAB ropy phenotype in GF baking is a promising feature because they can potentially act as hydrocolloids improving volume and texture and increasing dietary fibre content, which may acts as prebiotic (Gänzle 2014).

Lactic acid bacteria are widely known for their ability to inhibit pathogens by the production of antimicrobial compounds such as organic acids, oxygen peroxide and bacteriocins, which constitutes a sustainable alternative to food preservatives. *Listeria monocytogenes* and *B. subtilis* used as indicator organisms represent industrial environment contamination; they may cause spoilage of bakery products and constitute a health risk (Digaitiene *et al.* 2012). In this study, all detected antibacterial compounds were active after treatment with proteinase K evidencing their proteinaceous nature (data not shown). Antimicrobial compounds produced by *Ent. mundtii* CRL1896 and *Ent. casseliflavus* CRL1899 displayed antibacterial activity against *Listeria* and *Bacillus.* Bacteriocin production by enterococci has been reported since their encoding genes are widely disseminated among species from different origin (Feng *et al.* 2009; Özdemir *et al.* 2011). Rope spoilage of bakery products usually caused by *B. subtilis* and *Bacillus licheniformis* produce odour/colour changes and soft and sticky texture because of starch and proteins breakdown by microbial enzymes and EPS production (Menteş *et al.* 2007). In agreement with previous findings, leuconostoc and lactobacilli isolates where able to inhibit *Bacillus* species (Menteş *et al.* 2007; Masuda *et al.* 2011). Furthermore, we also detected antifungal activity against *A. oryzae* and *P. roqueforti* as previously reported by Ryan *et al.* (2008) and Oliveira *et al.* (2014). Mould growth is the most common microbial spoilage in bakery products leading to huge economic losses as well as reduced safety for consumers due to the production of mycotoxins. When the toxic biogenic amine production was investigated, *Lact. brevis* CRL1960 and CRL1951 strains were found as histamine producers; aminogenesis in this LAB species was also described for strains of wine origin (Landete *et al.* 2007).

The microbial resistance to antibiotics of clinical importance should be absent as it is required by EFSA as part of its Qualified Presumption of Safety (QPS)

assessment of bacteria deliberately introduced in the food chain. Accordingly, some of the LAB species here tested (*Lactococcus lactis, Leuc. mesenteroides, Lact. plantarum* and *Lact. brevis*) are included in the QPS list; their safety demonstration only entails resistance confirmation to antibiotics of clinical significance. All assayed *Lact. brevis* strains displayed high resistance to Amp, contrarily to those described for strains isolated from other fermented foods (Nawaz *et al.* 2011). Among *Leuconostoc* species, the found resistance to Chl, Kan and Amp is partially in agreement with Ammor *et al.* (2007) who stated that most leuconostoc species are usually resistant to Kan, Gen and Str but susceptible to Chl, Cli, Ery and Tet. Although some lactobacilli and leuconostoc strains in this study exhibited resistance to Van, it is mostly considered as an intrinsic feature (Ammor *et al.* 2007). Concerning enterococci, *Enterococcus hermanniensis* strains showed a low incidence of antibiotic resistance, whereas *Ent. casseliflavus* and *Ent. mundtii* were multiresistant strains. These results are in correlation with the prevalence of Gen and Str resistance and Van susceptibility reported for enterococcal species of vegetable origin (Abriouel *et al.* 2008). *Lactococcus lactis* CRL1895, *Leuc. mesenteroides* CRL1907, *Lact. plantarum* CRL1905 and CRL1906 were susceptible to all assayed antibiotics, thus they could be considered as safe to be used in food production.

In this study, a succession of LAB species occurred during continuous backslopping allowing *Lact. plantarum* and *Lact. brevis* to predominate and establish as a stable microbial association. *Leuc. mesenteroides* CRL1907 and *Lact. plantarum* CRL1905 were rationally selected based on their acidification capacity, ropy phenotype, antimicrobial activity and antibiotic susceptibility to be used as starter culture.

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Conflict of Interest

The authors declare that there is 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 LAB isolates biotypes and sequence information for RAPD-PCR obtained with primers M13b and XD9 during quinoa sourdoughs fermentation.

Table S2 Microbial species identification of DGGE bands after sequencing of the variable V3 region of 16S rRNA.