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Biopreservation potential of lactic acid bacteria from Andean fermented food of vegetal origin

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ABSTRACT

Microbial fermentations have long represented a way of natural biopreservation of raw materials, which frequently originated new food products. Among them, traditionally fermented products still manufactured by native populations all around the world are source of lactic acid bacteria (LAB) strains with high biotechnological potential. LAB are food grade microorganisms and therefore a good alternative to chemicals to be applied in food preservation. A total of 130 LAB isolates recovered from "chicha" and "tocosh", traditional fermented Andean products of vegetal origin, were screened for antimicrobial activities against spoiler fungi *Meyerozyma guilliermondii* CECT 1021 (synonym *Pichia guilliermondii*), *Penicillium roqueforti* CECT 2905^{NT}, *Aspergillus oryzae* CECT 2094^{NT} and *Aspergillus niger* CECT 2807 as well as against foodborne pathogens *Escherichia coli* O157:H7 CECT 5947, *Listeria innocua* CECT 910^{T} and *Salmonella enterica* subsp. *enterica* serovar Typhi CECT 4138. LAB isolates represented nine species and four genera that exhibited a general inhibition of food pathogens and were also active against *A. oryzae* and *M. guilliermondii* while a poor inhibition of *A. niger* and *P. roqueforti* was produced. Antifungal activity of cell free supernatants (CFS) from seven selected strains grown in MRS was confirmed against toxigenic fungi *Aspergillus parasiticus* CECT 2681, *Penicillium expansum* CECT 2278 and *Fusarium verticilloides* CECT 2987 and also on the three foodborne bacteria included in the study. Phenyllactic and 3,5-Di-O-caffeoylquinic acids were identified as the predominant bioactive compounds in CFS by QuEChERS extraction with LC-MS-LIT detection approach. Four out of seven strains free of antibiotic resistances involving *L. plantarum* M5MA1 and M9MM1 from chicha and *L. fermentum* T3M3 and *Lc. mesenteroides* T1M3 from tocosh showed high potential to be used as biopreservatives in food applications.

1. Introduction

Lactic acid bacteria (LAB) are present in a wide range of fermented food and beverages from traditional ancestral products to these days. They contribute to the transformation of raw materials during fermentation enriching their nutritional value, either by vitamins production, anti-nutrients reduction or increasing nutrient bioavailability (Ravyts, De Vuyst & Leroy, 2012; Tamang, Watanabe, & Holzapfel, 2016). However, lactic acid fermentations represent a food preservation strategy in which LAB transform higher alcohols and carbohydrates to mainly lactic acid. On this

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basis, they demonstrated an important role as biopreservatives in various foods and feed fermentation processes (Gobbetti, De Angelis, Corsetti, & Di Cagno, 2005). The use of LAB for their antibacterial properties is well known and has been extensively studied (Stoyanova, Ustyugova, & Netrusov, 2012). In addition, recently LAB antifungal potential is also being explored; which is of considerable interest in food and feeds where fungal spoilage is a problem (Crowley, Mahony, & Van Sinderen, 2013; Hugenholtz, 2013; Russo et al., 2016). Recently, traditionally fermented Andean food products such as "chicha" a traditional maize-based fermented beverage from Northwestern Argentina (Elizaquível et al., 2015) and "tocosh" fermented potatoes from the highlands of Central Peruvian Andes have been analysed and their LAB populations determined.

Because of the long history of consumption through traditional fermented foods, LAB have acquired the "Generally Recognized as Safe (GRAS)" status by the American Food and Drug Agency (FDA) and many LAB species are included in the "Qualified Presumption of Safety (QPS)" list established by the "European Food Safety Authority (EFSA)" (Leuschner et al., 2010). They are extensively used as starter cultures in the food industry, especially in dairy products (Leroy & De Vuyst, 2004). Additionally, as food grade components they constitute a good alternative to chemicals in food applications. The potential of LAB to extend the shelf life of food has been evaluated in bread, dairy and meat products, fresh fruits, vegetables and feed (Pawlowska, Zannini, Coffey, & Arendt, 2012).

Several compounds have been proposed as responsible for the antifungal activity of LAB, like organic acids, low molecular weight compounds, phenylacetic and fatty acids, cyclic dipeptides, proteinaceous compounds and other miscellaneous compounds e.g. lactones (Peyer et al., 2016). Research to-date has shown that single antimicrobial compounds do not completely exert the antifungal activity but the synergistic effect of several compounds may account for the observed activity as it has been suggested (Ndagano, Lamoureux, Dortu, Vandermoten, & Thonart, 2011). Spoilage fungi can grow on a broad variety of foods, *Aspergillus*, *Penicillium* and *Meyerozyma* genera being mostly isolated from cereals, vegetables, fruits and bakery products (Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013; Crowley et al., 2013; Valerio et al., 2009). Their growth leads to undesirable effects including the production of off-flavours and rottenness affecting quality and shortening shelf life. Blue mould caused by *Penicillium expansum* is one of the most serious fruit diseases in China and Europe (Qin & Tian, 2004) occurring during postharvest storage, shipping and marketing of fruits, dramatically reducing shelf life and causing serious economic losses. *Fusarium* species are widely distributed plant pathogens (Hefny, Attaa, Bayoumi, Ammar, & El-Bramawy, 2012) and *Fusarium verticillioides* is responsible for several major diseases in maize cultures, including stalk, root and ear rot (Reyes-Velázquez et al., 2011). Besides spoilage, growth of species like *Aspergillus parasiticus*, *F. verticillioides* and *P. expansum* can also lead to hazardous mycotoxin formation which is a great concern in food safety.

Analysis of antifungal compounds derived from LAB growth requires improved recovery procedures like the QuEChERS method, recently validated by Brosnan, Coffey, Arendt, and Furey (2014), combined with sensitive analytical techniques. Among them the most frequently applied are liquid chromatography linear ion trap quadrupole Orbitrap hybrid Fourier transform mass spectrometer (LC-FTMS) (Brosnan et al., 2014); high performance liquid chromatography (HPLC) system equipped with an ultra violet-diode array detector (UV/DAD) (Axel et al., 2015) or HPLC with an ultra violet–photo diode array (UV/PDA) detector (Peyer et al., 2016).

The main goal of food preservation is to stop microbial spoilage but also to prevent the growth of toxigenic foodborne microorganisms that constitute a health threat. In vegetable foods, the number of outbreaks associated with fresh produce has increased in the last years. Da Silva Felício et al. (2015) reported that the top ranking food/pathogen combination was leafy greens eaten raw and some fruits and *Salmonella* spp., followed by pathogenic *Escherichia coli* and fresh pods, legumes or grains, while *Listeria monocytogenes* have a ubiquitous nature persisting in food processing areas. Because of the LAB potential to produce natural antimicrobial agents, there is an increasing interest in the application of LAB as pathogen-antagonizing *in situ* strategy to keep the microbiological food safety (Cálix-Lara et al., 2014; Mills et al., 2011).

The aim of the present study was to screen LAB strains recovered from chicha and tocosh, traditional fermented Andean products, for antimicrobial activity and to further analyse cell free supernatant (CFS) of positive strains against toxigenic fungi and pathogenic bacteria. In addition, the antimicrobial compounds in CFS were investigated through a QuEChERS extraction coupled with the identification and quantification of the bioactive compounds by liquid chromatography (LC) associated to mass spectrometry linear ion trap (MS-LIT). As required for food applications, the antibiotic resistance profile of LAB strains was also determined.

2. Materials and methods

2.1. Microbial strains and growth conditions

LAB strains used in this study are part of the project "Microbiota of Andean Food: tradition for healthy products" (μ-Andes, ref. 247,650 FP7-PEOPLE-2009-IRSES) in which a huge number of isolates were recovered and characterized. In the present study, 130 strains recovered from two Latin American traditionally fermented products, chicha (68 strains, Elizaquível et al., 2015) and tocosh (62 strains) were included representing LAB species diversity and abundance in each product. They had been previously identified by molecular techniques as belonging to the species: *Enterococcus faecium* (15), *Lactobacillus brevis* (1), *L. casei* (10), *L. farciminis* (1), *L. fermentum* (1), *L. plantarum* (16), *L. sakei* (28), *Leuconostoc mesenteroides* (57) and *Pediococcus pentosaceus* (2) (Elizaquível et al., 2015).

LAB strains were routinely grown on de Man, Rogosa and Sharpe (MRS) broth (Conda, Madrid, Spain) at 30 °C for 24–48 h and stored for a long term at −20 °C in a 10% (w/v) dilution of the same broth medium supplemented with 20% (w/v) glycerol.

Indicator strains used for antimicrobial assays were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain): four spoiler fungi (*Meyerozyma guilliermondii* CECT 1021 (synonym *Pichia guilliermondii*), *Penicillium roqueforti* CECT 2905^{NT}, *Aspergillus oryzae* CECT 2094^{NT} and *A. niger* CECT 2807); three toxigenic fungi (*A. parasiticus* CECT 2681, *P. expansum* CECT 2278 and *Fusarium verticilloides* CECT 2987) and three bacterial foodborne pathogens (*Escherichia coli* O157:H7 CECT 5947, *Salmonella enterica* subsp. *enterica* serovar Typhi CECT 4138 (referred as *Salmonella* Typhi from now on), *Listeria innocua* CECT 910^{T} (as a *Listeria monocytogenes* surrogate). Fungi strains were grown on Malt broth (malt extract 2%, glucose 2% and mycopeptone 0.1%, w/v) at 25 °C for 5–7 days. Bacterial pathogens were grown overnight on Trypticasein Soy broth (TSB) or Trypticasein Soy Agar (TSA) (Conda, Madrid, Spain) at 37 °C.

2.2. Reagents and materials

The antifungal compounds 3-(4-hydroxy-3-methoxyphenyl) propanoic acid; Sinapic acid; 2-Deoxycytidine; Cyclo (L-His-L-Pro); Cyclo (L-Tyr-L Pro); Phenylpyruvic acid; *cis*-Caftaric acid; Protocatechuic acid hexoside; Caffeic acid derivative; Hydroxycinnamic acid derivative (*p*-cuomaric acid); Quercetin pentoside; Quinic acid

derivative; Caffeoylhexose-deoxyhexoside; 3,5-Di-O-caffeoylquinic acid were provided from Sigma-Aldrich (Dublin, Ireland). Phenyllactic acid (PLA) was obtained from BaChem (Weil am Rhein, Germany). All analytes had a purity of 95%. The HPLC grade solvents ethyl acetate (EA) and acetonitrile (ACN), drying agent magnesium sulphate ($MgSO_4$), C18, primary secondary amine (PSA), pancreatin, and sodium chloride (NaCl) were obtained from Sigma-Aldrich (Dublin, Ireland). Formic acid (FA) (99%) was bought from Fluka (Germany).

Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were filtered through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain).

2.3. Determination of antifungal activity

Bacterial activity against spoiler fungi *M. guilliermondii*, *P. roqueforti, A. niger* and *A. oryzae* was screened on 130 LAB strains as previously described by Magnusson, Ström, Roos, Sjögren, and Schnürer (2003). LAB strains were considered as positive when an inhibition zone of at least 10 mm around the bacterial growth was observed.

2.4. Determination of antibacterial activity

Antibacterial activity of 32 LAB strains out of 130, representing the species diversity in chicha and tocosh, was tested against *E. coli* O157:H7, *L. innocua* and *Salmonella* Typhi applying the procedure described by Gaudana, Dhanani, and Bagchi (2010). Briefly, 5 μL of LAB overnight cultures were spotted on soft agar (0.75% agar, w/v) TSA plates containing 1% inoculum of an overnight culture of the pathogen. The antibacterial activity was determined by presence of a growth inhibition halo around the tested strain.

2.5. Antimicrobial activity of cell free supernatants (CFS)

Eleven strains representing different species, food origin, antifungal and/or antibacterial activities were selected for CFS analysis. CFS recovered from 18 h cultures in MRS were prepared as previously described (Saladino, Luz, Manyes, Fernández-Franzón, & Meca, 2016). They were neutralized, concentrated by lyophilisation and re-suspended in fresh sterile MRS prior to be tested against toxigenic fungi *A. parasiticus*, *P. expansum* and *F. verticilloides* and bacterial pathogens *E. coli* O157:H7, *L. innocua* and *Salmonella* Typhi. Antifungal and antibacterial activities of CFS were determined by the agar well diffusion assay. They were also tested after hydrolyzation: CFS were treated with pancreatin at a final concentration of 30 IU/mg total protein and incubated at 37 °C for 48 h. For toxigenic fungi, 1 mL of 1×10^8 spores/mL of a 48 h fungus broth culture was gently spread onto Potato Dextrose Agar (PDA) plates, in triplicate. After drying, 100 μL of CFS were deposited in agar-excavated wells and incubated at room temperature for 20 days. For bacterial pathogens, 100 μL overnight cultures were spread onto TSA plates, CFS were added to agar wells as described for fungi, and plates were incubated at 37 °C for 48 h. A well with lyophilized MRS broth was included as negative control. The presence of an inhibition halo around the well was recorded as positive for antimicrobial activity.

2.6. Antibiotic resistance profile of LAB strains

Out of 130 LAB strains, 62 representing the species diversity in chicha and tocosh, were screened for antibiotics resistance. The antibiotics recommended by the European Food Safety Authority (EFSA, 2012) to identify bacterial strains with potential acquired resistance to antibiotics were analysed. The antibiotics tested were: ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline and vancomycin. The minimal inhibitory concentration (MIC) was determined by the broth microdilution method reported by the ISO 10,932/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.

2.7. Extraction, identification and quantification of presumptive antifungal active compounds by LC-MS/MS

The CFS that showed a strong antifungal activity were treated using the QuEChERS methodology for the extraction and quantification of the compounds responsible for the antifungal activity. In particular, 10 mL of the CFS were added to 10 mL of EA with 1% FA, 4 g of $MgSO_4$ and 1 g of NaCl and shaken for 1 min. The mixture was centrifuged for 10 min (3000 rpm) and the organic solvent supernatant was removed and added to a 15 mL plastic tube containing 150 mg of PSA, 150 mg of C18 and 885 mg of $MgSO_4$ and shaken for 1 min. The tube was then centrifuged for 10 min (3000 rpm). The solvent was dried under nitrogen flow at 35 °C (Turbovap, LV evaporator, Uppsala, Sweden), reconstituted to 1 mL with H_2O/ACN (90/10) and filtered (0.2 mm pore size filter), into a LC amber vials (1.5 mL capacity). Twenty microliters were injected onto the LC-MS system. This extraction was performed in triplicate for each CFS LAB culture.

The separation of the phenolic acids was achieved with a LC system (CMB-20A/LC-10AT, Shimadzu, Kioto, Japan) equipped with a Gemini C18 column (150 × 2.0 mm, 5 μm; Phenomenex, Madrid, Spain) with a guard column (Security Guard™ Gemini C18 cartridge AF0-8497; 4 × 3.0 μmID; Phenomenex, Madrid, Spain). The mobile phase composition was as follows. Solvent A: H_2O with 0.1% FA and solvent B: ACN with 0.1% FA. A gradient flow was performed to ensure separation of compounds (0 min-5% B; 5 min-10% B; 10 min-30% B; 20 min-30% B; 30 min-40% B; 35 min- 40% B; 40 min-95% B; 45 min-95% B, at a flow rate of 0.2 mL/min kept at a temperature of 30 °C. The injection volume was of 20 μL. The LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) was used to detect the presence of the compounds produced by the LAB in the MRS medium. The method was operated in "negative ionization" mode at 30,000 resolution with the following tune conditions: capillary temperature of 300 °C, capillary voltage −50 V, tube lens −110 V, sheath gas 45 arbitrary units and auxiliary gas 15 arbitrary units. Weekly calibration following manufacturer's instructions insured robust high mass accuracy spectrum (≤2 ppm) to confirm the presence of all compounds.

3. Results and discussion

3.1. Screening of chicha and tocosh LAB isolates for antimicrobial activity against spoiler fungi and foodborne pathogens

In the present study the antimicrobial activity against four-spoiler fungi *A. oryzae*, *A. niger*, *P. roqueforti* and *M. guilliermondii* was screened on 130 LAB strains recovered from chicha (68) and tocosh (62), two traditional Andean fermented food products. These fungi were selected as representative of those that can rapidly grow in a broad variety of cereal-based products (Oliveira, Zannini, & Arendt, 2014). No differences were observed in the antifungal activity pattern of strains from both products except for

Lc. mesenteroides; strains isolated from tocosh were more active than those from chicha (Table 1). Among the fungi tested, most of the strains inhibited *A. oryzae* and *M. guilliermondii*, both activities being frequently associated. LAB strains inhibiting at least one of the fungi belonged to the species *L. plantarum* (100%), *L. sakei* (96.43%), *L. casei* (90%), *E. faecium* (86.67%) and *Lc. mesenteroides* (84.21%). *A. niger* was poorly inhibited since only four strains from chicha, *Lc. mesenteroides* (2) and *L. plantarum* (2) showed antifungal activity, being *L. plantarum* M9MM2 the only strain of this species inhibitory only against *A. niger*, since the rest of them were also active against *A. oryzae*. In fact, *A. oryzae* was inhibited by the majority of the tested strains in coincidence with results reported for LAB species from quinoa, amaranth and wheat bran sourdoughs (Manini et al., 2016; Ruiz-Rodríguez, Vera Pingitore, Rollan, Cocconcelli et al., 2016; Ruiz-Rodríguez, Vera Pingitore, Rollan, Martos et al., 2016). Inhibition of *A. oryzae* had also been reported

Table 1
Antifungal activity of LAB isolates from chicha and tocosh against spoiler fungi (-, no inhibition; +, inhibition zone ≤ 1 cm; ++, > 2–3 cm; +++, >3 cm). Strains selected for CFS tests are shadowed.

	A. niger CECT 2807	*A. oryzae* CECT 2094^{NT}	*P. roqueforti* CECT 2905^{NT}	*M. guilliermondii* CECT 1021
Chicha				
E. faecium				
S4MM7, S4Y8, S5M2, S9MS2, S9Y7, S10MM6, S11Y7	−	++	−	−
S1Y5a, S1Y6, S3Y2, S3Y5, S10Y7, S11Y1	−	+	−	−
S8MS4, S9MS1	−	−	−	−
L. plantarum				
M2MG4	−	+	−	++
M5MA1	−	+++	++	++
M5MA4, M5MG1, M5MG6, M9MG3, M9MG5	−	++	−	++
M5MG2, M5MM1	−	++	−	−
M9MG6, M9MM1	−	++	−	+
M9MM2	+	−	−	−
M9MM4	+	+	−	−
M9MM5	−	+++	−	−
M9Y2	−	+++	−	+
Lc. mesenteroides				
M2Y6, M8MM2, M8Y1, M8Y2, S1M5b, S1M7b, S1MS4b, S4Y1, S5MM5, S5MS3, S10MS7b	−	++	−	−
M5MA2, M7MM1, M8Y5, M9Y3, S1Y7b, S4MM5, S10M4, S11sM5, S13MM5	−	−	−	−
M8MG2, M8MG6, S4MS3, S4MS4, S5M3	−	++	−	+
S1M6b	−	−	−	+
S1MS2b, S1MS6b, S3MS5	−	+++	−	−
S3MM7	−	+	−	−
S5M5, S5MM6	+	++	−	−
S5MS2, S11sMM1, M9MG2b	−	++	−	++
S10MS5b	−	−	−	+
P. pentosaceus				
S1M4	−	++	−	−
S11sM1	−	−	−	−
Tocosh				
L. brevis				
3T3R2	−	+	−	−
L. farciminis				
T3Y6	−	+++	−	+
L. fermentum				
T3M3	−	−	−	−
L. casei				
3T3MS1, 3T3MS2, 3T3MS7, 3T3MS10, 3T3MS12	−	+	−	−
3T3MS11, 3T3MS13, 3T3M2	−	++	−	−
3T3M3	−	−	−	−
3T3R1	−	++	−	++
L. sakei				
T2M3, T3MM1	−	+++	−	+
T3Y2, T3Y4, T3M1, T3MS2	−	+++	−	−
T3Y3, 2T2MS4, 3T1MS1	−	+	−	−
T3Y7	−	−	−	++
T3M2, T3MM4	−	+++	−	++
T3M7	−	+++	++	+
T3MM2	−	−	+	−
T3MS4, T3MS5, T2MM9, 2T2MM10, 2T3Y5, 2T2Y6, 2T3MS3, 2T3MS8	−	−	−	−
2T1MM5, 2T2M2	−	+	−	+
2T2MM5, 2T3MM10, 2T3MS6, 2T3MS9	−	−	−	+
Lc. mesenteroides				
T1M3, T1M4, T1M8, T2Y6, T2M1	−	+++	−	+
T1M5, T2Y7, T2M4	−	+++	−	+++
T2Y4, T2MM6, 2T1Y2, 2T1MM3, 2T2M5, 2T2M9, 2T2MS7	−	+	−	+
T2Y5, T2MM1, T2MS1	−	+++	−	−
T2MM3	−	+++	++	+
T2MM5	−	++	−	++
2T1Y7	−	−	−	+
2T3Y9	−	+	+	+

for LAB strains isolated from Malaysian fruits and fermented foods (Muhialdin & Hassan, 2011) which is in accordance with the results recorded in the present study for *L. brevis* (1) from tocosh and *P. pentosaceus* (1) from chicha. Regarding inhibition of *P. roqueforti* it was observed in only five strains belonging to the species *L. plantarum* (1) from chicha, *L. sakei* (2) and *Lc. mesenteroides* (2) from tocosh; these results being in accordance with those reported for semolina, quinoa sourdough and kimchi strains (Ruiz-Rodríguez, Vera Pingitore, Rollan, Cocconcelli et al., 2016; Valerio et al., 2009). Out of 130 LAB, only four strains, *L. plantarum* M5MA1 from chicha, *L. sakei* T3M7 and *Lc. mesenteroides* T2MM3 and 2T3Y9 from tocosh, showed antagonistic activity against *P. roqueforti, A. oryzae* and *M. guilliermondii*, these strains being highly valued because of their great potential as biopreservatives for food applications.

In addition, antibacterial activity against food pathogens of LAB species was also assayed. LAB are considered food-grade components and, therefore, there is a great interest in their potential as pathogen-antagonizing microorganisms for food preservation applications. Antibacterial activity has been widely described for *Lactobacillus*, *Leuconostoc* and *Pediococcus* against *Listeria*, *Salmonella* Typhi and *E. coli* (Cálix-Lara et al., 2014; Manini et al., 2016). *Listeria* species are ubiquitous and have been recovered from a large variety of food environments and food processing plants, from where they might enter the food chain. The presence of *Salmonella* Typhi and *E. coli* in low levels and/or other food spoilage microorganisms has been reported in wheat and flour (Sperber, 2007) as well as in products that are raw consumed (Cizeikiene et al., 2013; Da Silva Felício et al., 2015). In the present study, 32 out of 130 strains were tested for activity against three food-borne bacterial pathogens *E. coli* O157:H7, *L. innocua* and *Salmonella* Typhi (Table 2). Irrespective of the type of inhibitory compounds produced, LAB strains except one (*L. sakei* T3M7) inhibited the three pathogens, *Salmonella* Typhi being recorded as inhibited in a greatest extent. *E. faecium* strains moderately inhibited the three pathogens, *Lc. mesenteroides* from tocosh and *L. plantarum* from chicha showed the highest antibacterial activity against the three bacterial targets. Although, the main molecules which have been extensively studied as food antimicrobial agents are bacteriocins (Vignolo, Saavedra, Sesma, & Raya, 2012), other metabolites produced by LAB have shown to be highly inhibitory to food pathogens (Arena et al., 2016). Compared to LAB bacteriocins, which are mainly active against Gram-positive food pathogens such as *Listeria*, organic-acid producing LAB frequently exhibit a broader spectrum of antimicrobial action. Thus, the inhibition of *Salmonella* Typhi and *E. coli* O157:H7 by LAB strains particularly *L. plantarum*, may be assigned to organic acids production as reported by Arena et al. (2016).

Table 2
Antibacterial activity of 32 strains against three food-borne pathogens (+, inhibition zone ≤ 5 mm; ++, 6–9 mm; +++ ≥10 mm; -, no inhibition). Strains selected for CFS tests are shadowed.

	E. coli O157:H7 CECT 5947	*L. innocua* CECT 910^T	*Salmonella* Typhi CECT 4138
Chicha			
E. faecium			
S3Y2, S10Y7	+	+	+
S11Y1	+	++	+
L. plantarum			
M5MA1	+++[a]	++[a]	+++[a]
M9MG6	+++[a,b]	+++[a]	+++[a,b]
M9MM4	+++[a]	+++[a]	+++[a]
M9MM2, M9MM5	+++	+++	+++
M9MM1	++[a,b]	+++[a]	+++[a,b]
M9Y2	++[a]	+++[a]	+++[a]
Lc. mesenteroides			
M9MG2b	+++[a]	++[a]	+++[a]
M8MG2	+	++	+++
M8MG6	+	++	++
M8MM2	++	+	++
M8Y1	+	+	++
M8Y2, S4MS3, S5MM6	++	++	+++
S1MS2b	++	+	+++
S4Y1	–	++	++
S5M5	++	++	++
Tocosh			
L. farciminis			
T3Y6	++	+	+++
L. fermentum			
T3M3	+++[a]	+++[a]	+++[a]
L. casei			
3T3MS2	++	++	+++
L. sakei			
T3Y3	++	++	+++
T3M7	–	–	–
Lc. mesenteroides			
T1M3	+++[a,b]	+++[a]	+++[a,b]
T1M5, T2MM3	++	+++	+++
T2MM5	+++[a]	++[a]	+++[a]
T2MM6	+++[a]	++[a]	+++[a]
T2MS1	++	++	+++

CFS: Cell Free Supernatants (recovered from 18 h cultures in MRS), neutralized and concentrated.
[a] Activity of CFS.
[b] Lack of inhibitory activity using hydrolysed CFS.

3.2. Antibiotic resistance of LAB strains recovered from chicha and tocosh

Despite many LAB species have QPS status, aiming at food applications determination of antibiotics resistance is compulsory since LAB are commonly found among the resident microbiota of vertebrates and they might transfer resistances to pathogenic bacteria (Robredo, Singh, Baquero, Murray, & Torres, 2000). LAB strains tested in the present study belonging to the species *L. plantarum*, *L. sakei*, *L. casei*, *Lc. mesenteroides* and *P. pentosaceus* are good candidates for food applications since they are included in the QPS list (Leuschner et al., 2010). In the present study 62 out of 130 strains, representing the different species recovered from chicha and tocosh, were tested for antibiotic resistances according to EFSA's recommendation (International Standard ISO 10,932:2010/IDF 223:2010; EFSA, 2012). Table 3 summarizes the resistance patterns observed based on the MIC cut-off values established by EFSA for each species and antibiotic. Around 70% of strains tested were susceptible to the required antibiotics, irrespective of the origin, tocosh or chicha. The proportion of resistant strains was very similar for both food products, although multi-resistant strains (two or more antibiotics) were more frequent among tocosh isolates. Even when *Lactobacillus* were reported as usually sensitive to penicillins (Danielsen & Wind, 2003), resistance to ampicillin was the most frequently found among LAB strains in this study and it was accompanied by clindamycin-resistance in three *Lactobacillus* strains from tocosh (*L. brevis* and *L. sakei*). However, single resistances to ampicillin or tetracycline were also detected in some *Lc. mesenteroides* strains. Out of two *P. pentosaceus*, one showed resistance to erythromycin and the other to ampicillin and tetracycline. Surprisingly, all *E. faecium* strains were susceptible to the antibiotics tested, except one that showed resistance to vancomycin and ampicillin. Studies from last decades have consistently found vancomycin resistant enterococci isolated from different environments and it is a fact that resistant enterococci involve a risk of transferring antibiotic resistances from food to humans (Robredo et al., 2000).

Table 3
Antibiotic resistance profile of selected LAB strains from chicha and tocosh. Strains selected for CFS tests are shadowed.

Source of isolation/Species/Strains	Antibiotic resistance/ sensitivity[a]
Chicha	
E. faecium	
S3Y2, S8MS4, S9Y7, S10Y7, S11Y1	S
S11Y7	Amp/Van
L. plantarum	
M2MG4, M5MA1, M5MA4, M5MG1, M5MG2, M5MG6, M5MM1, M9MG3, M9MG5, M9MG6, M9MM1, M9MM2, M9MM5	S
M9MM4, M9Y2	Amp
Lc. mesenteroides	
M2Y6	Tet
M5MA2, M7MM1, M8MG6, M8Y1, M8Y5, M9MG2b, M9Y3, S4Y1, S5M3, S5M5	S
M8MG2, S4MS3, S10M4, S10MS5b, S11sMM1, S13MM5	Amp
S1M5b	Amp/Kan
P. pentosaceus	
S1M4	Ery
S11sM1	Amp/Tet
Tocosh	
L. brevis	
3T3R2	Amp/Cli
L. farciminis	
T2Y2	S
L. fermentum	
T3M3	S
L. casei	
3T3MS2, 3T3MS7, 3T3MS11, 3T3M2	S
L. sakei	
T3Y3, 3T1MS1	S
T3Y4, T3MM4	Amp/Cli
T3M1	Gen/Tet
T3M2	Gen
T3M7	Amp
T3MM1	S
Lc. mesenteroides	
T1M3, T1M5, T2Y6, T2MM3, T2MM6	S
T2MM5	Amp

[a] S, sensitive; Amp, ampicillin; Cli, clindamycin; Ery, erythromycin; Gen, gentamycin; Kan, kanamycin; Tet, tetracycline; Van, vancomycin.

3.3. Antimicrobial activity against mycotoxigenic fungi and bacterial pathogens. Preliminary identification of chemical nature of inhibitory agents

A selection of eleven strains including six *L. plantarum* (M5MA1, M9Y2, M9MM1, M9MM4, M9MG6, M9MG2), three *Lc. mesenteroides* (T1M3, T2MM5, T2MM6), *L. fermentum* (T3M3) and *L. sakei* (T3M7) was further tested for antifungal activity using their neutralized and concentrated CFS against three toxigenic fungi, *A. parasiticus*, *P. expansum* and *F. verticilloides* (Table 4). CFS from seven LAB strains resulted effective against the mycotoxigenic fungi studied, five of them (*L. plantarum* M5MA1, M9Y2, M9MM1, *Lc. mesenteroides* T1M3 and *L. fermentum* T3M3) inhibited the growth of *A. parasiticus*, *P. expansum* and *F. verticilloides*, whereas the other two (*L. plantarum* M9MM4, M9MG6) were effective only against *F. verticilloides*. The remaining four strains, *L. plantarum* M9MG2, *Lc. mesenteroides* T2MM5 and T2MM6, and *L. sakei* T3M7 did not exhibit antifungal activity against the assayed mycotoxigenic fungi.

In addition, CFS hydrolysed with pancreatin to exclude compounds of proteinaceous nature, corresponding to the seven inhibitory LAB strains, were tested against the toxigenic fungi and bacterial pathogens included in the study in order to compare with the antimicrobial activity of the non-hydrolysed CFS. Results showed that antifungal activity was not modified by the action of

Table 4
Antifungal activity detected in CFS obtained from selected chicha and tocosh LAB strains against the mycotoxigenic fungi (+, inhibition zone ≤ 10 mm; ++, 11–15 mm; +++ ≥17 mm; -, no inhibition). No differences were observed with hydrolysed CFS.

Strain	*A. parasiticus* CECT 2681	*P. expansum* CECT 2278	*F. verticilloides* CECT 2987
Chicha			
L. plantarum			
M9Y2	++	++	+++
M5MA1, M9MM1	++	++	++
M9MG6, M9MM4	–	–	++
Lc. mesenteroides			
M9MG2b	–	–	–
Tocosh			
L. fermentum			
T3M3	++	++	++
L. sakei			
T3M7	–	–	–
Lc. mesenteroides			
T1M3	+	+	+
T2MM5, T2MM6	–	–	–

CFS, Cell Free Supernatants (recovered from 18 h cultures in MRS), neutralized and concentrated.

CFS treated with the proteolytic enzyme since the same inhibition halos were observed for the corresponding LAB before and after CFS hydrolysis. Therefore, the possibility that LAB antifungal activity is due to proteinaceous compounds must be excluded. On the other hand, the antibacterial activity of hydrolysed CFS of three LAB strains (*L. plantarum* M9MG6, M9MM1 and *Lc. mesenteroides* T1M3) exhibited a lack of activity against pathogens (Table 2). This result indicates that a degradation of the inhibitory compound by pancreatin in the CFS must have occurred, thus the production of bacteriocins by LAB strains or the generation of other metabolism-derived proteinaceus compounds may be suggested.

However, it should not be discarded that antifungal effect might rely on the presence of non-hydrolysed small peptides since most antimicrobial peptides are composed by short amino acid chains (Dashper, Liu, & Reynolds, 2007). The low molecular weight of these peptides, the resulting higher exposure of the amino acids and their charges, and the formation of small channels in the lipid bilayer are responsible to their antimicrobial activity, these features promoting interactions between peptides and the membrane (Gómez-Guillén et al., 2010). Several studies available in the literature evaluated the antimicrobial properties of LAB fermented products against different mycotoxigenic fungi. Indeed, the control of *F. culmorum* growth during fermentation of a malt-based substrate by active metabolites released by *L. brevis* R2D and *L. plantarum* FST1.7 was reported (Peyer et al., 2016); the highest inhibition was due to the former while the latter only reduced the mould growth. In contrast, in the present study *L. plantarum* strains (five out of six) inhibited the growth of *F. verticilloides*. In another study, the antifungal effect of *L. plantarum* CRL778 isolated from sourdough was reported (Gerez, Torino, Rollán, & Font de Valdez, 2009) which is in accordance to the results that are here reported.

3.4. Determination and quantification of the antifungal compounds

In the present study, CFS that showed antifungal activity were analysed by the combination of QuEChERS extraction with LC-MS-LIT detection allowing a fine identification and quantification of up to 15 compounds. The QuEChERS extraction had been previously used by Brosnan et al. (2014) as a novel technique to improve the recoveries of antifungal compounds from LAB cultures. The method was validated on LAB strains that showed particularly strong antifungal activity (*L. plantarum*, *L. amylovorus*, *Weissella cibaria*),

Table 5
Bioactive antifungal compounds identified in the CFS obtained from strains *L. plantarum* M5MA1 and M9MG6, and *L. fermentum* T3M3 (expressed in mg/L).

Antifungal Compounds	*L. plantarum* M5MA1	*L. plantarum* M9MG6	*L. fermentum* T3M3
Phenyllactic acid	90.00 ± 5.63	65.00 ± 5.13	_
3-Propanoic acid	7.50 ± 2.24	_	_
Sinapic acid	_	9.50 ± 1.27	_
2-Deoxycytidine	15.00 ± 3.48	15.50 ± 2.57	_
Cyclo (L-His-L Pro)	4.45 ± 1.52	_	_
Cyclo (L-Tyr-L Pro)	6.50 ± 1.84	0.95 ± 0.34	10.00 ± 2.64
Phenylpyruvic acid	4.55 ± 1.69	9.50 ± 1.69	0.85 ± 0.25
cis-Caftaric acid	_	1.37 ± 1.28	_
Protocatechuicacidhexoside	_	1.65 ± 0.89	0.95 ± 0.52
Caffeic acid derivative	5.50 ± 1.74	_	_
Hydroxycinnamic acid derivative	_	24.00 ± 3.56	_
Quercetinpentoside	20.00 ± 3.28	_	_
Quinic acid derivative	22.50 ± 3.94	_	_
Caffeoylhexose-deoxyhexoside	2.95 ± 1.21	2.75 ± 1.84	2.75 ± 0.91
3,5-Di-O-caffeoylquinic acid	24.15 ± 4.27	22.50 ± 5.32	18.00 ± 5.63

providing an increase in the number of compounds detected (both known and unknown). The compounds detected were confirmed by LC-FTMS and included 1,2-dihydroxybenzene, DL-þ-hydroxyphenyllactic acid, 4-hydroxybenzoic acid, 3,4-Dihydroxyhydrocinnamic acid, vanillic acid, caffeic acid, 3-(4-hydroxyphenyl)−propionic acid, PLA, *p*-coumaric acid, 3-(4-hydroxy-3-methoxyphenyl) propanoic acid, benzoic acid, ferulic acid, salicylic acid, hydrocinnamic acid, methylcinnamic acid. In another study, HPLC-UV/DAD technique was successfully applied to analyse the antifungal compounds present in extracts of sourdough fermented with antifungal *L. amylovorus* DSM19280, allowing to determine higher concentrations of antifungal compounds in the antifungal active sourdough than in the non-antifungal fermented product (Axel et al., 2015). More recently, Peyer et al. (2016) quantified phenolic acids released by *L. plantarum* FST1.7 and *L. brevis* R2D in barley malt extract and modified MRS medium using a QuEChERS method coupled with a HPLC-UV/PDA system. By this approach, from a total of thirteen phenolic acids analysed, five were present in unfermented wort (4-hydroxybenzoic acid, hydrocaffeic acid, vanillic acid, *p*-coumaric acid, and ferulic acid) whereas the 3-phenyllactic acid was the only phenolic compound produced by each strain in both substrates, with *L. plantarum* FST1.7 being the highest producer in wort. In MRS medium a strain-dependent release of phenolic acid compounds was observed, in particular phloretic acid and hydroferulic acid for *L. plantarum* FST1.7 and benzoic acid for *L. brevis*. In this study, three out of seven antifungal LAB strains (*L. plantarum* M5MA1, M9MG6 and *L. fermentum* T3M3) produced phenolic acids (Table 5). *L. plantarum* M5MA1 produced eleven different phenolic compounds at concentrations ranging from 2.95 mg/L for caffeoylhexose-deoxyhexoside to 90.00 mg/L for PLA, whereas five bioactive compounds were produced by *L. fermentum* T3M3 with concentrations between 0.85 mg/L for phenylpyruvic acid to 18.00 mg/L for 3,5-Di-O-caffeoylquinic acid. In addition, *L. plantarum* M9MG6 produced ten phenolic acids, the lowest and the highest concentrations were obtained for cyclo-(L-Tyr-L-Pro) 0.95 mg/L and PLA 65.0 mg/L, respectively. Among phenolic compounds, cyclo-(L-Tyr-L-Pro), phenylpiruvic acid, caffeoyl hexose-deoxyhexoside and 3,5-Di-O-caffeoylquinic acid were identified in all three assayed LAB strains with mean concentrations of 5.82, 4.97, 2.82 and 21.55 mg/L, respectively. PLA was the highest produced both by *L. plantarum* M5MA1 and *L. plantarum* M9MG6, followed by 3,5-Di-O-caffeoylquinic acid, hydroxycinnamic acid derivative, quinic acid derivative and quercetin pentoside. Concentrations of the other phenolic compounds were lower than 20.00 mg/L for the three strains. Compared to previous studies, among LAB species *L. plantarum* was mostly found as producer of antifungal compounds (Axel et al., 2015). In addition, identified and quantified phenolic compounds produced by different strains showed PLA and 3-propanoic acids in common with our results (Gerez et al., 2009; Valerio et al., 2009).

4. Conclusions

LAB strains isolated from chicha and tocosh, two vegetable traditional fermented Andean products, demonstrated to be effective at inhibiting several toxigenic and non-toxigenic fungi such as *Aspergillus*, *Penicillium* and *Fusarium* as well as food pathogens. Chemical nature of antimicrobial agents (bacteriocins and/or antifungal molecules) were preliminarily identified by using the CFS of LAB strains concentrated and/or hydrolysed with pancreatine. The QuEChERS extraction with LC-MS-LIT detection allowed the identification and quantification of up to 15 antimicrobial compounds. The chicha strains, *L. plantarum* M5MA1 and M9MM1, and tocosh strains *L. fermentum* T3M3 and *Lc. mesenteroides* T1M3 have potential as antimicrobial agents by inhibiting bacterial foodborne pathogens and spoiler fungi as well as by their CFS activity against toxigenic fungi. They are free of antibiotic resistances and therefore suitable for food applications. To highlight, *L. plantarum* M5MA1 that, in addition, inhibits *P. roqueforti*, *A. oryzae* and *M. guilliermondii*, it is the best candidate as an alternative to preservatives in the food industry.

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