Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2017;27:289–298
DOI: 10.1159/000480542

Published online: November 23, 2017

Lactic Acid Bacteria from Andean Grain Amaranth: A Source of Vitamins and Functional Value Enzymes

Silvana L. Carrizo[a] Cecilia E. Montes de Oca[a] María Elvira Hébert[a]
Lucila Saavedra[a] Graciela Vignolo[a] Jean Guy LeBlanc[a]
Graciela Celestina Rollán[a, b]

[a]Centro de Referencia para Lactobacilos (CERELA) – CONICET, San Miguel de Tucumán, and [b]Universidad del Norte Santo Tomás de Aquino (UNSTA), Yerba Buena, Argentina

Keywords
Lactic acid bacteria · Amaranth · Riboflavin · Folate · Phytase

Abstract
Amaranth is a rediscovered pseudocereal with high nutritional properties. Lactic acid fermentation can increase the functional and nutritional value of foods. The aims of this study were to isolate and evaluate the functionality of lactic acid bacteria (LAB) from amaranth. LAB strains ($n = 29$) isolated from amaranth sourdough and grains included *Lactobacillus* (*L.*) *plantarum* ($n = 8$), *L. rhamnosus* ($n = 6$), *Enterococcus* (*E.*) *mundtii* ($n = 4$), *E. hermanniensis* ($n = 3$), *E. durans* ($n = 1$), *Enterococcus* sp. ($n = 1$), *Leuconostoc* (*Lc.*) *mesenteroides* ($n = 3$), and *Lc. mesenteroides* subsp. *mesenteroides* ($n = 3$). Only 21% of the strains showed the ability to synthesize capsular exopolysaccharides or display ropiness and only 8 strains showed amylolytic activity. *L. plantarum* CRL 2106 and *E. durans* CRL 2122 showed the highest phytase activity, which is of importance for mineral bioavailability. *L. plantarum* CRL 2106 and CRL 2107 and *Lc. mesenteroides* subsp. *mesenteroides* CRL 2131 synthesized the highest concentrations of B_2 and B_9 vitamin (140–250 ng/mL). This study demonstrates the potential of LAB to improve the nutritional and functional values of pseudocereal-derived foods.

Introduction

Amaranth is a gluten-free grain that belongs to the *Amaranthaceae* family [Alvarez-Jubete et al., 2009]. The amaranth grain (AG) is rich in starch like conventional cereals, and thus it is often referred to as a "pseudocereal" [Bressani, 2003]. Amaranth is an ancient crop cultivated in different countries in South and Central America, Africa, India, and Asia, which has been rediscovered in the last 30 years [Dini et al., 2012]. AG has been reported to have higher amounts of minerals (Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}) compared to wheat [Alvarez-Jubete et al., 2009; Venskutonis and Kraujalis, 2013]. Nevertheless, the bioavailability of these nutrients can be affected by antinutri-

KARGER

E-Mail karger@karger.com
www.karger.com/mmb

Dr. Graciela Celestina Rollán
CERELA – CONICET
Chacabuco 145
San Miguel de Tucumán T4000ILC (Argentina)
E-Mail rollan@cerela.org.ar

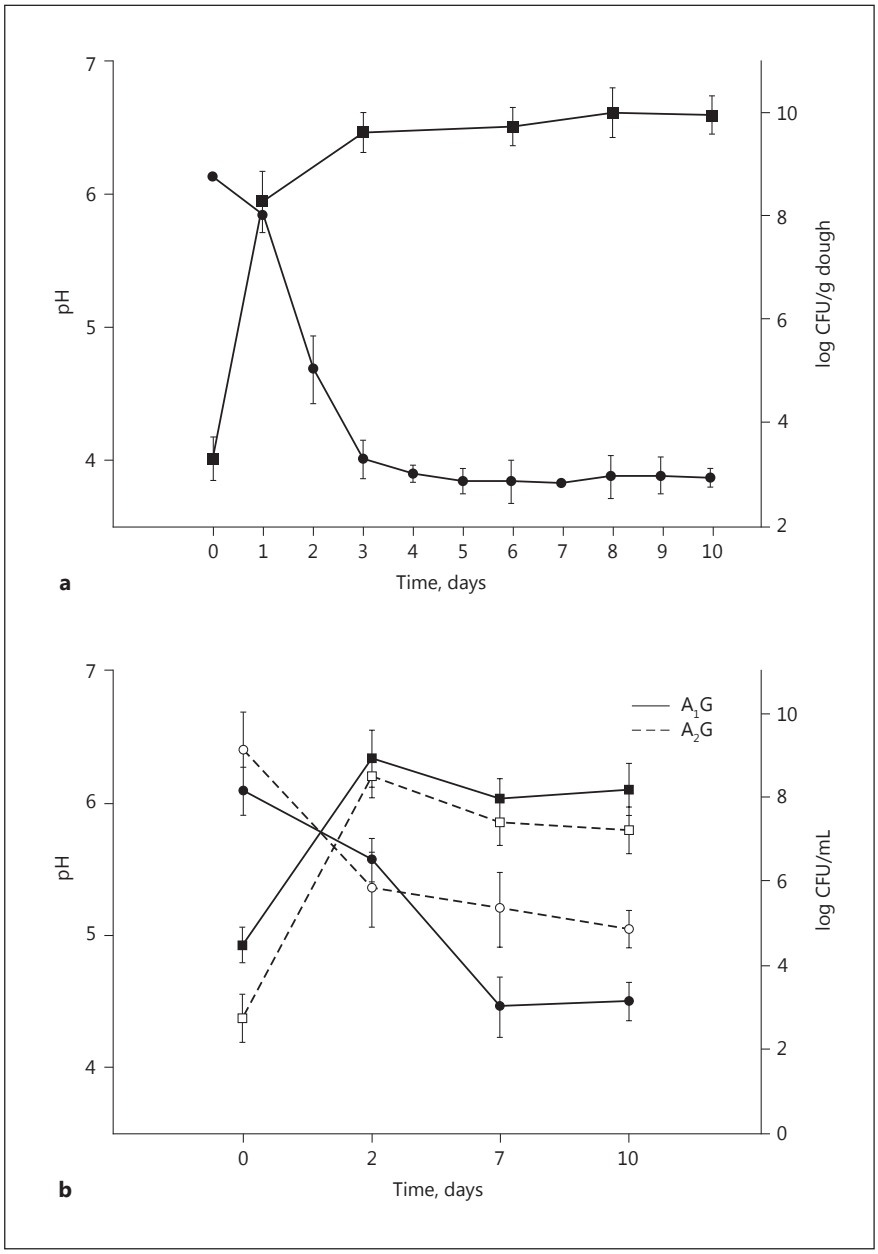

Fig. 1. Viable cell-counts (squares) and pH values (circles) of SAS during fermentation (**a**) or from LAB isolated from A_1G and A_2G (**b**). Results are expressed as means ± SD.

tive compounds. In this sense, amaranth is rich in phytic acid (PA; myo-inositol hexakisphosphate, 21.1 μmol/g), which can form complex multivalent cations such as minerals and inhibit their intestinal absorption [Sanz-Penella et al., 2012]. The negative effect of PA can be reduced by phytase, an enzyme capable of sequentially dephosphorylating PA. Since microorganisms are the main source of phytases, there is increasing biotechnological potential for their use in PA-containing foods [Lopez et al., 2000].

Lactic acid bacteria (LAB) are a heterogeneous group of microorganisms with GRAS (Generally Recognized as Safe) status that have traditionally been associated with food fermentation. These bacteria produce a wide variety of metabolites and compounds during fermentation, such as organic acids, aroma compounds, antimicrobial substances, exopolysaccharides (EPS), bioactive peptides, and useful enzymes, among others [Hugenholtz, 2008]. Although LAB are usually auxotrophic for several vita-

mins, some strains have the ability to synthesize a few B-group vitamins, suggesting that the adequate use of selected strains may increase the concentration of these vitamins in fermented foods, increasing their nutritional value. Selection of starter cultures within the autochthonous microbiota is strongly recommended since indigenous cultures ensure better performance compared to allochthonous/commercial strains [Di Cagno et al., 2013].

Several review articles have been published on different aspects of amaranth, including the study of its composition, processing, uses, properties, and potential beneficial health effects [Alvarez-Jubete et al., 2010; Caselato-Sousa and Amaya-Farfán, 2012]. In contrast, only a few reports about the characterization of LAB from amaranth sourdough and their use as starter culture are available [Sterr et al., 2009; Vogelmann et al., 2009; Jekle et al., 2010].

Thus, the aim of this study was isolation, identification, and partial characterization of lactic microbiota from amaranth (grains and sourdough) in order to select suitable LAB strains as starter cultures for the elaboration of amaranth-derived foods with improved nutritional, technological, and functional values. This was accomplished by selecting strains that have the capacity to produce bioactive substances (such as vitamins, ropiness [R], and capsular EPS [CPS]) and produce functional value enzymes (phytase and amylase).

Results

LAB Isolation and Identification

Microbiological analysis of the lactic microbiota of SAS (spontaneous amaranth sourdough) fermentation showed an increase from 2×10^3 to 5×10^9 CFU/g dough at 24 h, which remained constant until the end of the fermentation (Fig. 1a). In the AG, LAB in A_1G increased from 3.0×10^4 to 6.8×10^8 CFU/mL while in A_2G it increased from 4.6×10^2 to 2.8×10^8 CFU/mL at 48 h of fermentation (Fig. 1b).

The presumptive LAB isolated from SAS ($n = 30$) and AG ($n = 60$) on MRS-5 at 30°C were subjected to preliminary trials for identification. From of the total ($n = 90$) isolates, 70 were considered putative LAB (Gram positive and catalase negative); microscopic analyses showed the presence of cocci (58.6%) and bacilli (41.4%).

Genotypic Characterization by RAPD-PCR Analysis

Presumptive LAB (70 colonies) were analyzed by RAPD-PCR using 2 primers, i.e., XD9 and M13b. Strains with identical RAPD patterns were considered the same strain. The results showed 29 different patterns or RAPD profiles. The 16S rRNA of the representative strain of each RAPD profile was partially sequenced using the PLB16 and MLB16 primers (data not shown). The respective species were determined by BLAST comparison of the obtained sequences with the nucleotide database of the National Center for Biological Information (NCBI). The twenty-nine different patterns corresponded to different strains of *Lactobacillus* (*L.*) *plantarum* ($n = 8$), *L. rhamnosus* ($n = 6$), *Enterococcus* (*E.*) *mundtii* ($n = 4$), *E. hermanniensis* ($n = 3$), *E. durans* ($n = 1$), *Enterococcus* sp. ($n = 1$), *Leuconostoc* (*Lc.*) *mesenteroides* subsp. *mesenteroides* ($n = 3$), and *Lc. mesenteroides* ($n = 3$). All of the different LAB strains were deposited in the CERELA Culture Collection (CRL).

Acidification Capacity

The acidification capacity of the 29 LAB strains was evaluated in sterile flour extract for 72 h. *L. rhamnosus* CRL 2139 and *L. plantarum* CRL 2079, 2092, 2107, and 2108 isolated from SAS, as well as *Lc. mesenteroides* CRL 2132 and *E. durans* CRL 2122 isolated from AG, reduced the medium pH to values lower than 5.0 after 8 h, while the remaining strains reached that pH after 24 h. The final pH values (72 h) were between 3.70–3.90, except for *L. rhamnosus* CRL 2139 and *E. mundtii* CRL 2094 isolated from SAS and *E. mundtii* CRL 2136, *E. hermanniensis* CRL 2134, *Lc. mesenteroides* subsp. *mesenteroides* CRL 2131, and *Lc. mesenteroides* CRL 2121 isolated from AG, the medium pH value of which was was higher than 4.0 (data not shown).

Amylolytic Activity

Owing to their relatively high starch content, amaranth appears to be an important eco-niche for screening and isolation of amylolytic LAB (ALAB). LAB (29 strains) isolated from SAS and AG were evaluated for amylolytic activity in modified MRS agar containing 1% soluble starch. Of the total, 8 strains, i.e., *L. plantarum* CRL 2079, 2080, 2092, and 2106 from SAS and *E. hermanniensis* CRL 2134 and 2136, as well as *E. mundtii* CRL 2137 and *Enterococcus* sp. CRL 2138 from AG, showed amylolytic activity (Table 1).

Ropiness and CPS Formation

The LAB strains isolated from SAS and AG were evaluated for CPS and R production. Of the total studied strains, 20.7% were CPS^+ and R^+, 38% were CPS^+ and R^-, and 17.2% were CPS^- and R^+ while the remaining 24%

Table 1. Characteristics of LAB isolated from amaranth

LAB	Source	CPS	Ropiness	Amylolytic activity	Phytase activity, U/mL
L. plantarum CRL 2079	SAS	+	+	+	$699 \pm 47^{h-j}$
L. plantarum CRL 2080	SAS	+	+	+	$636 \pm 48^{h-j}$
L. plantarum CRL 2082	SAS	–	+	–	$333 \pm 49^{c-e}$
L. rhamnosus CRL 2081	SAS	–	–	–	$234 \pm 1^{b-e}$
L. rhamnosus CRL 2139	SAS	–	+	–	135 ± 41^{j}
L. rhamnosus CRL 2090	SAS	–	+	–	$300 \pm 3^{b-e}$
L. rhamnosus CRL 2091	SAS	–	+	–	$291 \pm 13^{b-e}$
L. plantarum CRL 2092	SAS	+	+	+	$577 \pm 65^{g-i}$
L. rhamnosus CRL 2093	SAS	–	–	–	$355 \pm 25^{e, f}$
L. rhamnosus CRL 2104	SAS	–	–	–	0^{a}
L. plantarum CRL 2105	SAS	–	+	–	$648 \pm 9^{h-j}$
L. plantarum CRL 2106	SAS	+	+	+	$730 \pm 25^{i, j}$
L. plantarum CRL 2107	SAS	+	+	–	$530 \pm 42^{f-h}$
E. mundtii CRL 2094	SAS	+	–	–	$287 \pm 47^{b-e}$
L. plantarum CRL 2108	SAS	+	+	–	$136 \pm 7^{a, b}$
Lc. mesenteroides CRL 2121	AG	–	–	–	$373 \pm 61^{e, f}$
E. durans CRL 2122	AG	+	–	–	$1{,}041 \pm 48^{k}$
Lc. mesenteroides subsp. *mesenteroides* CRL 2123	AG	–	–	–	$358 \pm 25^{e, f}$
Lc. mesenteroides CRL 2124	AG	+	–	–	$370 \pm 5^{e, f}$
E. hermanniensis CRL 2125	AG	–	–	–	$387 \pm 14^{e, f}$
Lc. mesenteroides subsp. *mesenteroides* CRL 2131	AG	+	–	–	$406 \pm 18^{e-g}$
Lc. mesenteroides CRL 2132	AG	+	–	–	$338 \pm 37^{d, e}$
E. hermanniensis CRL 2133	AG	+	–	–	$255 \pm 53^{b-e}$
E. hermanniensis CRL 2134	AG	+	–	+	$156 \pm 17^{a-c}$
E. mundtii CRL 2135	AG	+	–	–	$685 \pm 8^{h-j}$
E. mundtii CRL 2136	AG	+	–	+	$588 \pm 30^{h-j}$
E. mundtii CRL 2137	AG	+	–	+	$598 \pm 8^{h-j}$
Lc. mesenteroides subsp. *mesenteroides* CRL 2140	AG	+	–	–	0^{a}
Enterococcus sp. CRL 2138	AG	–	–	+	$167 \pm 7^{a-d}$

LAB, lactic acid bacteria. SAS, spontaneous amaranth sourdough; AG, amaranth grain; CPS, capsular exopolysaccharide. Symbols indicate the capacity (+) or not (–) of the strains to degrade starch. Data presented as mean ± standard deviation. [a–k] Different letters in the same column indicate significant differences ($p < 0.05$).

were CPS^- and R^-. These results demonstrated that the ability to synthesize CPS or to display R is strain dependent (Table 1).

Antinutritional Factor Degradation: Phytase Activity

Phytase activity was widely distributed in all of the assayed species; the highest phytase activities were determined in *L. plantarum* CRL 2106 (730 ± 25 U/mL) and *E. durans* CRL 2122 (1,041 ± 48 U/mL) isolated from SAS and AG, respectively. For the remaining LAB strains, the phytase activity varied between 135 U/mL and 699 U/mL (Table 1).

Riboflavin and Folate Production

Riboflavin (B_2) and folate (B_9) production was determined in LAB isolated from SAS and AG. *L. plantarum* was the species that produced the highest vitamin concentration, and the SAS strains *L. plantarum* CRL 2106 and *L. plantarum* CRL 2107 produced the highest concentrations of B_9 (138 ± 7.5 ng/mL) and B_2 (158 ± 25 ng/mL), respectively. Other strains synthesized variable vitamins levels, except for *L. rhamnosus* CRL 2093 which did not produce B_2 (Fig. 2a). The extracellular production of riboflavin and folate in LAB isolated from SAS was always higher than the intracellular production.

Ten out of 14 LAB isolated from AG produced B_9, and 3 of these strains, i.e., *Lc. mesenteroides* subsp. *mesen-*

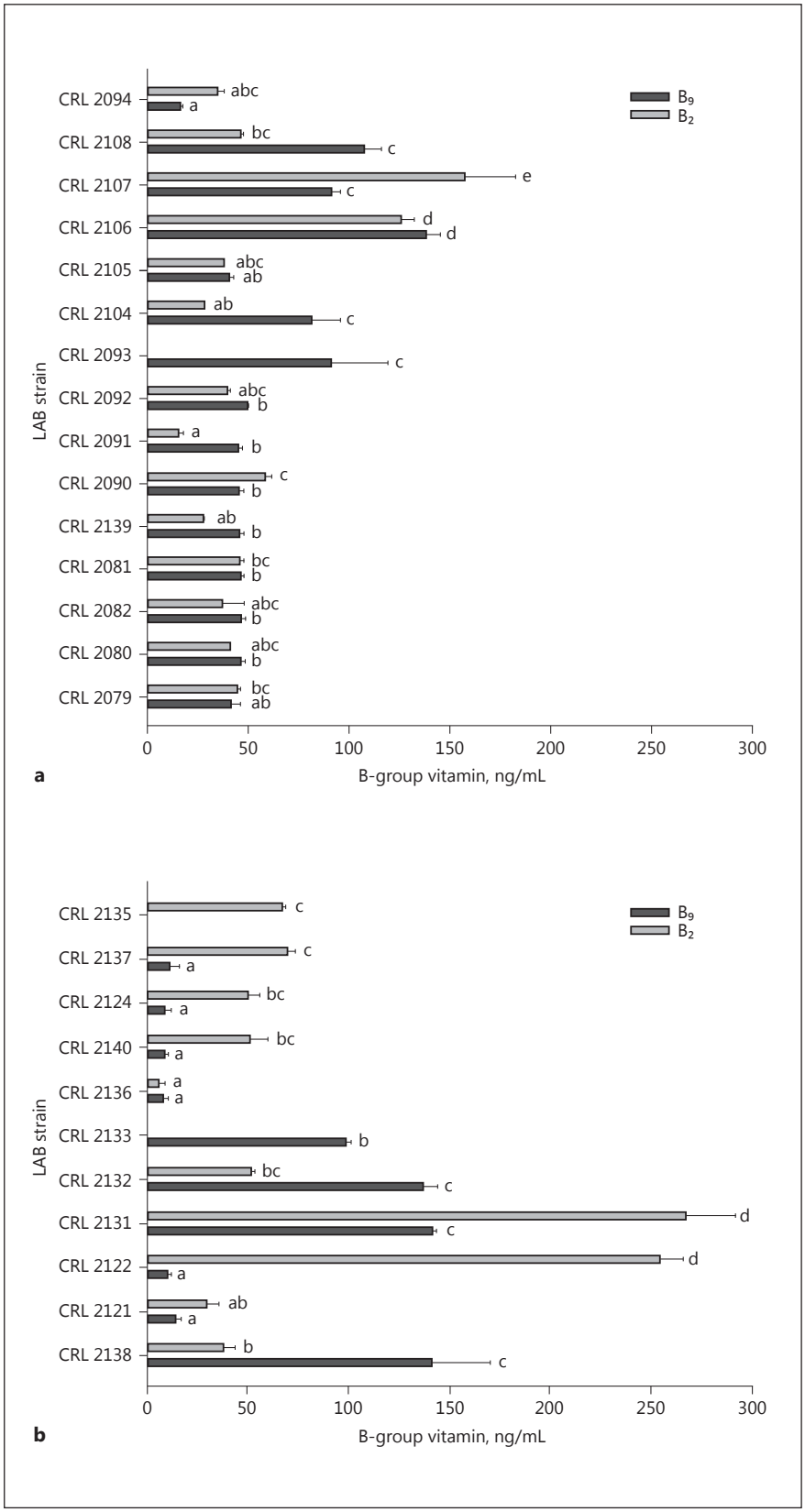

Fig. 2. Total riboflavin (B_2) and folate (B_9) concentration (ng/mL) produced by LAB isolated from amaranth sourdough (**a**) or grains (**b**). Data are presented as means ± SD. Different letters for the same vitamin indicate significant differences ($p < 0.05$). LAB, lactic acid bacteria.

teroides CRL 2131, *Lc. mesenteroides* CRL 2132, and *Enterococcus* sp. CRL 2138, synthetized the highest concentrations (>130 ng/mL; Fig. 2b); CRL 2131 was the best producer (142.2 ± 2.1 ng/mL). Variable concentrations (9–100 ng/mL) were produced by the other LAB strains. *Lc. mesenteroides* subsp. *mesenteroides* CRL 2131 and *E. durans* CRL 2122 produced the highest total riboflavin concentrations (>250 ng/mL).

Discussion

In this study, 29 LAB strains isolated and identified from amaranth (sourdough and grain) were subjected to characterization to be used as a potential starter culture in the production of amaranth-derived foods with higher nutritional and functional values.

Characterization of LAB from Amaranth

Sourdough fermentation is a traditional process employed since ancient times in baking; it has the potential to exploit the technological, nutritional, functional, and sensory features of wheat and nonwheat flours [Katina et al., 2005; Arendt et al., 2011; Gänzle, 2014].

ALAB from Amaranth

Polysaccharides constitute the main compositional part of amaranth seeds, with starch being the main component (67.3%) in this fraction. Although most LAB are unable to degrade starch due to lack of the enzyme amylase, some strains exhibit this enzymatic activity and are qualified as ALAB. Only few strains of lactobacilli exhibit amylase activity [Gänzle and Follador, 2012] but ALAB have been reported to be found in different tropical starchy fermented foods, made especially from roots such as cassava and sweet potato or grains such as maize, sorghum, and rice [Ramos et al., 2015; Smerilli et al., 2015]. In this study, strain-specific LAB amylolytic activity from amaranth was demonstrated. Certain LAB isolated from SAS, with most belonging to *L. plantarum*, have the ability to degrade starch.

LAB Producing R and CPS

Gluten-free baking is often characterized by a low bread volume and a poor texture due to the lack of network-forming gluten proteins [Gallagher et al., 2003]. EPS, high-molecular-weight carbohydrate polymers, are produced by certain LAB strains during sourdough fermentation and they have the potential to replace hydrocolloids in gluten-free sourdoughs [Schwab et al., 2008]. EPS-producing LAB are industrially important microorganisms in the production of functional foods and they are frequently used as starter cultures or coadjutants to develop fermented foods such as yoghurt, cheese, and cereal-based products [Zannini et al., 2016]. In this study, LAB strains isolated from SAS and AG were evaluated for CPS and R production. The results showed that these properties are strain dependent. *L. rhamnosus* CRL 2081, 2093, and 2014 were R^- CPS^-, while *L. rhamnosus* CRL 2139, and CRL 2090 and 2091 were R^+ and CPS^-. Six strains of *L. plantarum* showed R production in addition to formation of CPS. These results open the door exploitation of these EPS- and CPS-producing strains for the development of amaranth-based fermented foods with quality and acceptability similar to those of products made with gluten-containing flours.

Functional Properties of LAB from Amaranth

Antinutritional Factor Degradation: Phytase Activity

Whole-grain products, such as whole-grain breads, have been recommended because of their high content of several minerals (e.g., Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, and Mn^{2+}). However, the major parts of minerals in grains are complexly bound to PA (such as phytate), consequently reducing their bioavailability [Kumar et al., 2010]. The PA content of GF flours/foods is an important factor to consider, especially for celiac patients who suffer from micronutrient deficiencies. GF flours/ingredients have variable concentrations of phytate, i.e., rice, 0.12%; pearl millet, 0.25%; amaranth, 0.47%; teff, 0.70%; lupin, 0.77%; corn, 0.92%; oats, 1.13%; quinoa, 1.18%; and soybean, 1.33% [Arendt et al., 2011]. Sanz-Penella et al. [2012] showed that replacing wheat flour with whole amaranth flour as a bread-making ingredient (up to 40/100 g) affected the phytate concentration (up to 1.20 μmol/g dry matter [dm]) of bread with respect to controls, which did not detectable values, thus reducing the mineral bioavailability. The application of phytase-producing LAB as starter cultures has been proposed as a food grade alternative. Selected strains of LAB species from sourdough fermentation as well as from an African pearl millet fermentation have been reported to be capable of degrading PA [López et al., 2000; De Angelis et al., 2003; Songré-Ouattara et al., 2008]. In our study, LAB strains belonging to different species isolated from SAS and AG partially degraded PA, and *L. plantarum* CRL 2106 (SAS) and *E. durans* CRL 2122 (AG) were the strains that showed the highest phytase activity (i.e., 730 ± 25 and 1,041 ± 48 U/mL, respectively). These activity values are higher than those reported in the study of Anastasio et al. [2010],

where the highest phytase activity value (710 U/mL) was determined for *L. plantarum* H5. The bacteriocin-producing strains *Pediococcus pentosaceus* KTU05–8 and KTU05–9, isolated from spontaneous rye sourdough, showed a phytase activity of 32 and 54 U/mL, respectively, under conditions similar to leavening of bread dough (pH 5.5 and 30°C) [Cizeikiene et al., 2015]. Also, García-Mantrana et al. [2014] reported that purified phytases from intestinal bifidobacteria reduced the high level of phytates in breads made with the addition of whole amaranth flour.

These results highlight that phytases as produced during food fermentation by LAB represents an attractive strategy to produce whole grain products (from cereals and pseudocereals) with enhanced nutritional quality. In addition to B vitamin production by amaranth-fermenting LAB, this seems to be the first report on their phytase activity.

Additionally, phytase, proteolytic, and amylolytic activities may be influenced to a greater degree by the pH profile of biological acidification during the fermentation period in contrast to the rather abrupt effect caused by the deliberate addition of acidic compounds. The optimum activity of these enzymes, which play a significant role in changes in dough constituents, is achieved at pH values of 5.0–6.0, 4.0–5.0, and 3.6–6.2 for phytase and proteolytic and amylolytic enzymes, respectively [Poutanen et al., 2009; Gänzle, 2014]. As demonstrated in the present study, certain LAB strains isolated from both SAS as AG can reduce the pH to values below 6.0 at the beginning of fermentation, which would greatly improve enzymatic activities.

Production of Folate and Riboflavin

Grain products contain only low amounts of group B vitamins due to their loss during processing methods. Previously, it was demonstrated that staple foods produced from amaranth contained total folate contents of 35.5 μg/100 g dm in bread, 36.3 μg/100 g dm in cookies, and 38.9 μg/100 g dm in noodles, whereas when wheat was used, breads contained 12.0 μg/100 g [Schoenlechner et al., 2010]. The riboflavin content in AG flour is in the range of 0.29–0.32 mg/100 g. In general, significant reductions of all vitamins take place during processing, which affect the nutritional value of the products [Dini et al., 2012]. The production of fermented food products with high levels of B vitamins increases their commercial and nutritional value and eliminates the need for fortification [Burgess et al., 2009]. Some LAB strains can synthesize B vitamins. Different strategies have been applied to improve the microbial production of vitamins during fermentation [Sybesma et al., 2006]. In this study, 29 LAB isolated from amaranth were evaluated for their capacity to produce B_2 and/or B_9. From these strains, 79% were able to grow in the absence of riboflavin and 86% without folates. These values are similar to those obtained previously with strains isolated from goat milk and cheeses, where 84% were capable of producing at least one of these vitamins [Da Silva et al., 2016]. *L. plantarum* CRL 2106 and CRL 2107, both isolated from SAS, produced relatively high B_9 and B_2 amounts. *Lc. mesenteroides* subsp. *mesenteroides* CRL 2131, isolated from AG, synthesized the highest vitamin concentrations (142.2 ± 2.1 ng/mL B_9 and 267.5 ± 24.4 ng/mL B_2). These results are higher than those reported in a recent study where 40 strains isolated from wheat produced folates between 30 and 70 ng/mL [Salvucci et al., 2016]. Moreover, *L. plantarum* CRL 2106 and 2107 produced higher concentrations of folate than the values recently reported by strains of this species [Laiño et al., 2014]. Folate and riboflavin production was also strain dependent, as was the case for strains isolated from other ecological niches such as dairy products, cereals, fermented vegetables, etc. [LeBlanc et al., 2013]. To our knowledge, this is the first study showing that fermenting LAB strains isolated from amaranth can produce riboflavin and folate.

Conclusions

The results obtained in this work led us to select *L. plantarum* CRL 2106 and *E. durans* CRL 2122, isolated from SAS and AG, respectively, as a potential starter culture for preparation of amaranth-based foods as they: (i) grow in amaranth doughs, causing their acidification; (ii) produce elevated B-group vitamin (folate and riboflavin) concentrations; (iii) produce EPS; and (iv) possess important enzymatic activities that are not only technologically but also nutritionally relevant as they may degrade antinutritional factors such as phytate.

This work contributes to the fundamental knowledge of the LAB metabolism during fermentation of nonconventional cereal crops. Fermentation of amaranth with selected LAB could be used as a strategy to improve the nutritional and functional value of foods by producing nutritional compounds and reducing undesirable ones. However, the strains selected in this study must be evaluated in fermented amaranth foods in order to confirm whether the results obtained in vitro are conserved in this food matrix.

Materials and Methods

LAB Isolation

Sourdough was prepared as described previously by Carrizo et al. [2016]. Every 24 h, samples were drawn to determine the pH using a portable pH meter equipped with a penetration probe (Sartorius PT-10; Germany) and for microbiological analysis. Ripe sourdough samples (50 g) were stored at –20°C for further analysis.

To analyze the bacterial count in the SAS, 5-g portions were suspended in 45 mL of sterile saline solution (0.85% weight/volume of sodium chloride) and mixed for 1.5 min in a Stomacher machine (Stomacher 400; Seward, UK). For isolation and counting of LAB from sourdough was performed as previously described by Carrizo et al. [2016]. Gram staining was performed and the catalase activity and the cell morphology of the isolates were determined. Presumptive LAB isolates (Gram positive and catalase negative) were selected for identification and characterization and stored at –80°C in glycerol (20% volume/volume, final concentration) for further studies.

In addition, LAB were isolated from 2 AG varieties, i.e., A_1G and A_2G [Carrizo et al., 2016]. The morphology of tentative LAB isolates (cells and colonies) was observed to determine purity and Gram staining was performed and the catalase activity determined. All Gram-positive and catalase-negative isolates were stored at –80°C in glycerol (20% final concentration) for further analysis.

Differentiation and Identification of LAB Using Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction

Cultures (5 mL) grown in MRS were prepared with single colonies and incubated at 30°C for 16 h. Two milliliters of these cultures were centrifuged (10 min, 3,000 *g*) and the resulting cell pellets were used for DNA isolation according to Pospiech and Neuman [1995].

Randomly amplified polymorphic DNA (RAPD)-PCR was carried out with a MycyclerTM thermal cycler (BIO-RAD Laboratories, Inc.) using the single primers XD9 (5′-GAAGTCGTCC-3′) and M13b (5′-GAGGGTGGCGGTTCT-3′) [Carrizo et al., 2016]. RAPD-PCR profiles were analyzed using Scientific Image Processing (ImageJ 1.47v) software. LAB isolates were considered different when the RAPD-PCR profiles were not the same based on the profiles obtained using the primers M13b or XD9.

One representative strain of each RAPD profile was subjected to sequencing of the first 550 bp of the 16S rRNA gene with the primers PLB16 (5′-AGAGTTTGATCCTGGCTCAG-3′) and MLB16 (5′-GGCTGCTGGCACGTAGTTAG-3′) according to the protocol described by Hébert et al. [2000]. After purification, products were sequenced using an ABI 3130 DNA sequencer (Applied Biosystems, Foster, CA, USA). rRNA gene sequence alignments were performed using the multiple sequence alignment method [Edgar, 2004] and identification queries were fulfilled by a BLAST search [Altschul et al., 1990] in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) and in the Ribosomal Database Project [Cole et al., 2009].

Characterization of LAB

Acidification Capacity

The acidification capacity by LAB in vitro was determined in sterile flour extract [Alfonzo et al., 2013]. LAB cultures were individually inoculated (1% volume/volume) in 20 mL of sterile flour extract, and the tubes were incubated for 72 h at 30°C. The pH measurements were taken at 0, 4, and 8 h and then 24, 48, and 72 h after inoculation.

Screening of ALAB

To determine the capacity of LAB from SAS and AG to qualitatively hydrolyze starch in vitro, the method of Lee et al. [2001] was applied with modifications [Carrizo et al., 2016]. The starch hydrolysing strain *L. amylovorus* CRL 887 was used as a positive control.

R and CPS Formation

Overnight cultures (5 μL) of LAB were spotted on MRS agar plates and incubated at 30°C for 48 h; the ability to form ropy colonies was assessed by observing a mucoid appearance when touching the colonies with a sterile toothpick [Ruas-Madiedo and De Los Reyes-Gavilán, 2005]. CPS formation was determined by the India ink negative-staining technique from an active culture grown in liquid MRS for about 16–24 h [Mozzi et al., 2001].

Phytase Activity: Preparation of Cell-Free Extracts and Phytase Assay

LAB were preliminary inoculated in Chalmers broth [Vanos and Cox, 1986] with 1% (weight/volume) of sodium phytate (Sigma-Aldrich) and incubated at 30°C to the exponential growth phase. Preparation of the cell-free extract was carried out as described previously by Carrizo et al. [2016]. Phytase activity was determined by measuring the concentration of inorganic phosphate released from sodium phytate [Shimizu, 1992]. Absorbance was measured at $A_{700\ nm}$ using a microplate reader (VERSAmax tuneable microplate reader; Molecular Devices, USA). Results were compared to a standard curve prepared with inorganic phosphate (K_2HPO_4). One unit of phytase activity was defined as the amount of phytase that liberates 1 nmol · mL^{-1} inorganic phosphate per min from a 3 mM Na-phytate solution at pH 5.0 and a temperature of 45°C. The volumetric activity of intracellular phytase was determined as described previously by Nuobariene et al. [2011].

Riboflavin (B_2) and Folate (B_9) Vitamin Production

The selection of vitamin (B_2 and B_9)-producing LAB was determined previously [Carrizo et al., 2016]; vitamin production was evaluated in those strains able to grow in the absence of the corresponding vitamin.

Folate concentrations (intra- and extracellular and total) were determined by a previously described microbiological assay using *L. rhamnosus* NCIMB 10463 as an indicator strain [Carrizo et al., 2016].

Intracellular, extracellular, and total riboflavin concentrations were likewise determined using *L. rhamnosus* ATCC 7469 as an indicator strain grown in riboflavin-free medium [Carrizo et al., 2016].

Statistical Analysis

All values were expressed as means ± SD. Statistical analyses were performed with the software package Infostat using ANOVA GLM followed by Tukey's post hoc test, and $p \leq 0.05$ was considered statistically significant.

Acknowledgements

The authors acknowledge the financial support of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), and the Ministerio de Ciencia, Tecnología e Innovación Productiva (MINCyT).

Disclosure Statement

The authors declare that they have no conflict of interests.

References

Alfonzo A, Ventimiglia G, Corona O, Di Gerlando R, Gaglio R, Francesca N, Moschetti G, Settanni L: Diversity and technological potential of lactic acid bacteria of wheat flours. Food Microbiol 2013;36:343–354.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic local alignment search tool. J Mol Biol 1990;215:403–410.

Alvarez-Jubete L, Arendt E, Gallagher E: Nutritive value and chemical composition of pseudocereals as gluten-free ingredients. Int J Food Sci Nutr 2009;60:240–257.

Alvarez-Jubete L, Arendt E, Gallagher E: Nutritive value of pseudocereals and their increasing use as functional gluten-free ingredients. Trends Food Sci Technol 2010;21:106–113.

Anastasio M, Pepe O, Cirillo T, Palomba S, Blaiotta G, Villani F: Selection and use of phytate-degrading LAB to improve cereal-based products by mineral solubilization during dough fermentation. J Food Sci 2010;75:M28–M35.

Arendt EK, Moroni A, Zannini E: Medical nutrition therapy: use of sourdough lactic acid bacteria as a cell factory for delivering functional biomolecules and food ingredients in gluten free bread. Microb Cell Fact 2011;10:1–9.

Bressani R: Amaranth; in: Encyclopedia of Food Sciences and Nutrition. Cambridge, Academic Press, 2003, pp 166–173.

Burgess CM, Smid EJ, van Sinderen D: Bacterial vitamin B_2, B_{11} and B_{12} overproduction: an overview. Int J Food Microbiol 2009;133:1–7.

Carrizo L, Montes de Oca CE, Laiño JE, Suarez NE, Vignolo G, LeBlanc JG, Rollán G: Ancestral Andean grain quinoa as source of lactic acid bacteria capable to degrade phytate and produce B-group vitamins. Food Res Int 2016;89:488–494.

Caselato-Sousa VM, Amaya-Farfán J: State of knowledge on amaranth grain: a comprehensive review. J Food Sci 2012;77:R93–R104.

Cizeikiene D, Juodeikiene G, Bartkiene E, Damasius J, Paskevicius A: Phytase activity of lactic acid bacteria and their impact on the solubility of minerals from wholemeal wheat bread. Int J Food Sci Nutr 2015;66:736–742.

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen A, McGarrell DM, Marsh T, Garrity GM: The ribosomal database project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 2009;37:D141–D145.

Da Silva FFP, Biscola V, LeBlanc JG, de Melo Franco BDG: Effect of indigenous lactic acid bacteria isolated from goat milk and cheeses on folate and riboflavin content of fermented goat milk. LWT Food Sci Technol 2016;71:155–161.

De Angelis M, Gallo G, Corbo MR, McSweeney PL, Faccia M, Giovine M, Gobbetti M: Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CB1. Int J Food Microbiol 2003;87:259–270.

Di Cagno R, Coda R, De Angelis M, Gobbetti M: Exploitation of vegetables and fruits through lactic acid fermentation. Food Microbiol 2013;33:1–10.

Dini C, García MA, Viña SZ: Non-traditional flours: frontiers between ancestral heritage and innovation. Food Funct 2012;3:606–620.

Edgar RC: Muscle: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32:1792–1797.

Gallagher E, Gormley T, Arendt E: Crust and crumb characteristics of gluten free breads. J Food Eng 2003;56:153–161.

Gänzle MG: Enzymatic and bacterial conversions during sourdough fermentation. Food Microbiol 2014;37:2–10.

Gänzle MG, Follador R: Metabolism of oligosaccharides and starch in lactobacilli: a review. Front Microbiol 2012;3:1–15.

García-Mantrana I, Monedero V, Haros M: Application of phytases from bifidobacteria in the development of cereal-based products with amaranth. Eur Food Res Technol 2014;238:853–862.

Hébert E, Raya R, Tailliez P, de Giori GS: Characterization of natural isolates of *Lactobacillus* strains to be used as starter cultures in dairy fermentation. Int J Food Microbiol 2000;59:19–27.

Hugenholtz J: The lactic acid bacterium as a cell factory for food ingredient production. Int Dairy J 2008;18:466–475.

Jekle M, Houben A, Mitzscherling M, Becker T: Effects of selected lactic acid bacteria on the characteristics of amaranth sourdough. J Sci Food Agric 2010;90: 2326–2332.

Katina K, Arendt E, Liukkonen KH, Autio K, Flander L, Poutanen K: Potential of sourdough for healthier cereal products. Trends Food Sci Technol 2005;16:104–112.

Kumar V, Sinha AK, Makkar HP, Becker K: Dietary roles of phytate and phytase in human nutrition: a review. Food Chem 2010;120:945–959.

Laiño JE, del Valle MJ, de Giori GS, LeBlanc JG: Applicability of a *Lactobacillus amylovorus* strain as co-culture for natural folate bio-enrichment of fermented milk. Int J Food Microbiol 2014;191:10–16.

LeBlanc JG, Milani C, de Giori GS, Sesma F, van Sinderen D, Ventura M: Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr Opin Biotechnol 2013;24:160–168.

Lee H, Gilliland S, Carter S: Amylolytic cultures of *Lactobacillus acidophilus*: potential probiotics to improve dietary starch utilization. J Food Sci 2001;66:338–344.

Lopez HW, Ouvry A, Bervas E, Guy C, Messager A, Demigne C, Remesy C: Strains of lactic acid bacteria isolated from sour doughs degrade phytic acid and improve calcium and magnesium solubility from whole wheat flour. J Agric Food Chem 2000;48:2281–2285.

Mozzi F, Torino MI, de Valdez GF: Identification of exopolysaccharide-producing lactic acid bacteria. Food Microbiol Protoc 2001;14:183–190.

Nuobariene L, Hansen A, Jespersen L, Arneborg N: Phytase-active yeasts from grain-based food and beer. J Appl Microbiol 2011;110:1370–1380.

Pospiech A, Neumann B: A versatile quick-prep of genomic DNA from gram-positive bacteria. Trends Genet 1995;11:217–218.

Poutanen K, Flander L, Katina K: Sourdough and cereal fermentation in a nutritional perspective. Food Microbiol 2009;26:693–699.

Ramos CL, de Sousa ES, Ribeiro J, Almeida TM, Santos CC, Abegg MA, Schwan RF: Microbiological and chemical characteristics of tarubá, an indigenous beverage produced from solid cassava fermentation. Food Microbiol 2015;49:182–188.

Ruas-Madiedo P, De Los Reyes-Gavilán C: Invited review: methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. J Dairy Sci 2005;88:843–856.

Salvucci E, LeBlanc JG, Pérez G: Technological properties of lactic acid bacteria isolated from raw cereal material. LWT Food Sci Technol 2016;70:185–191.

Sanz-Penella JM, Laparra JM, Sanz Y, Haros M: Bread supplemented with amaranth (*Amaranthus cruentus*): effect of phytates on in vitro iron absorption. Plant Foods Hum Nutr 2012;67:50–56.

Schoenlechner R, Wendner M, Siebenhandl-Ehn S, Berghofer E: Pseudocereals as alternative sources for high folate content in staple foods. J Cereal Sci 2010;52:475–479.

Schwab C, Mastrangelo M, Corsetti A, Gänzle M: Formation of oligosaccharides and polysaccharides by *Lactobacillus reuteri* LTH5448 and *Weissella cibaria* 10M in sorghum sourdoughs. Cereal Chem 2008;85:679–684.

Shimizu M: Purification and characterization of phytase from *Bacillus subtilis* (natto) N–77. Biosci Biotechnol Biochem 1992;56:1266–1269.

Smerilli M, Neureiter M, Wurz S, Haas C, Frühauf S, Fuchs W: Direct fermentation of potato starch and potato residues to lactic acid by *Geobacillus stearothermophilus* under nonsterile conditions. J Chem Technol Biotechnol 2015;90:648–657.

Songré-Ouattara L, Mouquet-Rivier C, Icard-Vernière C, Humblot C, Diawara B, Guyot JP: Enzyme activities of lactic acid bacteria from a pearl millet fermented gruel (ben-saalga) of functional interest in nutrition. Int J Food Microbiol 2008;128:395–400.

Sterr Y, Weiss A, Schmidt H: Evaluation of lactic acid bacteria for sourdough fermentation of amaranth. Int J Food Microbiol 2009;136:75–82.

Sybesma W, Hugenholtz J, De Vos WM, Smid EJ: Safe use of genetically modified lactic acid bacteria in food: bridging the gap between consumers, green groups, and industry. E J Biotechnol 2006;9:1–25.

Vanos V, Cox L: Rapid routine method for the detection of lactic acid bacteria among competitive flora. Food Microbiol 1986;3:223–234.

Venskutonis PR, Kraujalis P: Nutritional components of amaranth seeds and vegetables: a review on composition, properties, and uses. Compr Rev Food Sci Food Saf 2013;12:381–412.

Vogelmann SA, Seitter M, Singer U, Brandt MJ, Hertel C: Adaptability of lactic acid bacteria and yeasts to sourdoughs prepared from cereals, pseudocereals and cassava and use of competitive strains as starters. Int J Food Microbiol 2009;130:205–212.

Zannini E, Waters DM, Coffey A, Arendt EK: Production, properties, and industrial food application of lactic acid bacteria-derived exopolysaccharides. Appl Microbiol Biotechnol 2016;100:1121–1135.