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RESEARCH PAPER

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Artisanal tanneries: Potential application of inoculants formulated with lactic acid bacteria

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In artisanal tanneries, the skins are immersed in cereals fermented by natural microbial flora in order to reduce the pH of the skin, an essential condition for carrying out the final step. The environmental thermal variation alters the fermentation process and affects the quality of the final product. The aim of this work was to isolate lactic acid bacteria from cereals mixture fermented in an artisanal tannery and to evaluate *in vitro* the acidifying activity of the strains as a first step for the formulation of a starter culture. In most samples, a prevalence of cocci (95%) was observed with respect to bacilli. The best acidifying strains were identified by phenotypic and genotypic techniques as *Enterococcus faecium* CRL 1943 (rapid acidification at 37 °C) and *Leuconostoc citreum* CRL 1945 (high acidifying activity at 18 °C). In addition, the biomass production of the selected strains was analyzed at free and controlled pH (bioreactors 1.5 L). The production of biomass was optimal at controlled pH, with a higher growth (0.5–1.1 log units). Both strains were compatible, allowing their inclusion in a mixed culture. These lactic strains could contribute to the systematization of the tanning process.

KEYWORDS
artisanal tanning, lactic inoculants, purging stage

1 | INTRODUCTION

Tanning is the process of transforming the animal skin (a natural renewable resource) to leather (a market material used in the manufacture of a wide range of products) [1]. The leather industry is one of the oldest in Argentina and provides work places to the country due to the abundance of raw material (mainly skin cattle) and the quality achieved with international recognition. Currently, the export business of leather focuses on larger tanneries; they must be able to satisfy the volume and quality required by international trade. Artisanal tanneries located in different parts of the Argentine are using skins from sheep and goats of small producers as raw material. The artisanal tanning process consists in various steps such as washing skins, liming, depilation, lime removal by washing, purging, and tanning. During purging, the skins are immersed in a naturally fermented cereal mixture. After a while, the pH of skins decreased to 5.0, a condition that is required for the final steps. The cereal mix is left to ferment for 12–24 h at room temperature (18–37 °C) or until pH 4.5–5.0. In the province of Tucumán, artisanal tanneries use wheat flour and corn bran which contain starch as mordant and substrate for fermentation. At present, under these homemade working conditions, the cereal fermentation is quite variable and affects the quality of the leather.

Lactic acid bacteria (LAB) are used as starter cultures in food processing to induce changes in their properties such as texture modification, aroma, nutritional improvement [2], or food preservation. The main applications are as lactic starters

Abbreviations: LAB, lactic acid bacteria; TTA, titratable acidity.

in dairy and bakery products [3], but also in alcoholic drinks [4]. In tannery, LAB could be use to achieve the required lowering pH by lactic acid fermentation, under controlled conditions. The aim of this study was isolate LAB from the fermented cereal mixture in artisanal tanneries and to evaluate *in vitro* the acidifying activity of the strains as a first step for the formulation of a starter culture for tanneries, in order to improve the process. In addition, the production of selected LAB in economic media was evaluated using bioreactors to minimize costs.

2 | MATERIALS AND METHODS

2.1 | LAB isolation

Samples from fermented cereal mixture (wheat flour, corn bran, sodium chloride) were taken at an artisanal tannery (San Pedro de Colalao, Tucuman, Argentina) in sterile jars collectors and stored at −20 °C until processing. After thawing, samples were microscopically analyzed and the pH was measured. Aliquots (20 ml) of each sample were diluted into 180 ml of a 1% (w/v) sterile peptone solution. Serial decimal dilutions (10–2 to 10–5) were made and spread on MRS and LAPTg agar and incubated at 30 and 37 °C under microaerophilic conditions for 5 days (all culture media used were added with 0.5% of cycloheximide to prevent the yeast growth). Gram positive and catalase negative isolates were considered LAB. Pure cultures were preserved at −20 °C for further examination.

2.2 | Acidification activity and LAB selection

Twenty-three LAB were assayed for acidification activity at 18 and 37 °C (thermal variations [cold and hot periods] of the region throughout the year), 24 h in MC broth. MC medium contains (g/L): 20 glucose, 10 yeast extract, 10 skim milk, 3.6 Na_2HPO_4, 5.6 KH_2PO_4, 0.038 $MnSO_4$, and 0.05 $MgSO_4$. For this purpose, strains were overnight revitalized in the same media at 18 and 37 °C and then they were inoculated at 0.1% (v/v) in 100 ml of MC broth. Samples were taken at 4, 6, 8 y, 24 h; acidification activity was determined by pH and total titratable acidity (TTA) measurements; Dornic solution (0.1 N NaOH) was used to titrate acids and phenolphthalein as pH indicator. Results were expressed in milliliters of Dornic solution needed to achieve a pH of 8.3–8.6. Each experiment was replicated three times and results were expressed as the mean of measurements.

2.3 | Genotypic identification of selected strains

The genotypic identification of selected strains was determined on the basis of sequencing of variable region (V1) of the 16S rDNA as previously described by Hébert et al. [5]. Oligonucleotides for PCR reactions were: PLB16, 5′-AGAGTTTGATCCTGGCTCAG-3′; and MLB16, 5′-GGCTGCTGGCACGTAGTTAG-3′. PCR amplification consisted of 30 cycles of 30 s/94 °C, 30 s/50 °C, and 1 min/72 °C. Final PCR products were purified using a commercial kit (AccuPrep® PCR Purification Kit; Bioneer Corporation, Genbiotech, Buenos Aires, Argentina) and subjected to sequencing (Servicio de Secuenciación, Centro Científico-Tecnológico CONICET, Tucumán, Argentina). Resulting sequences were analysed on line using the NCBI BLAST algorithms (National Center of Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

2.4 | Phenotypic identification of selected strains

Phenotypic identification of selected strains was performed by API 50 CH system (Biomérieux), according to manufacturer's instructions. Results were analyzed according to the biochemical profiles registered in the APIweb® database (bioMerieux).

2.5 | Production of selected LAB using bioreactors

Batch fermentations were performed in a 2 L bioreactor (INFORS HT, Switzerland). MC medium (1.5 L, pH 6.5) was inoculated (1% v/v) with selected cultures. During fermentation, temperature was maintained at 37 °C; agitation speed at 150 rpm; and pH was adjusted to 6.0 (*Enterococcus faecium* CRL 1943) or 5.5 (*Leuconostoc citreum* CRL 1945) with a 20% (v/v) NH_4OH solution. Fermentations were performed for 24 h. Samples were aseptically withdrawn every hour during 12 and at 24 h of incubation and mixed uniformly with a vortex before diluting. Cell viability was determined by plating appropriate dilutions of the cultures in MRS agar (Britania, Buenos Aires, Argentina, plus 15 g L^{-1} agar). Plates were incubated at 37 °C for 48 h and colony-forming units (CFU ml^{-1}) were determined. Results were expressed as log CFU ml^{-1}. pH measurements were determined with a digital pH meter (Altronix TPX 1, New York, USA). Ethanol, lactic and acetic acids were measured by HPLC as previously described by Gerez et al. [6] using an Aminex HPX-87H ion-exclusion column (ISCO 2350 model, 300×7.8 mm^2, Bio-Rad, Hercules, CA, USA). Metabolite concentrations were expressed as g L^{-1}. Sugars consumption was also evaluated by HPLC according to Ortiz et al. [7] using an Aminex HPX-87P column (Bio-Rad Laboratories Inc.; San Francisco, CA, USA). All data were analyzed using the Eurochrom Basic Edition for Windows software.

2.6 | Strains compatibility

Compatibility of selected strains was evaluated by the plate diffusion assay [8]. Overnight cultures grown in MRS were washed twice with sterile saline solution and suspended at the original volume. Plates were prepared by pouring 10 ml MRS soft agar (MRS plus 7 g L^{-1} agar) containing 60 µl of one cell suspension. After overlay solidification, 5 mm diameter wells were made by using sterilized plastic cylinders. Wells were inoculated with 60 µl of supernatants from the other culture strain. After incubation at 37 °C for 16 h, clear inhibition zones were observed.

3 | RESULTS

3.1 | Isolation and selection de LAB from fermented cereals mixture

In order to select a starter to be used as inoculant, samples from fermented cereal mixture used in an artisanal tannery were analyzed. Microscopic observation showed round cocci; pH of the samples were 5.0 ± 0.5. Twenty-three gram-positive, catalase-negative cocci were presumptively classified as LAB. Acidification capacity (ΔpH, ΔTTA) of LAB was taken into account in order to select strains. This activity was evaluated at 18 and 37 °C according to mean values of the thermal variations recorded in the region throughout the year. Initial fermentation pH was 6.5 ± 0.1. After 24 h at 37 °C, ΔpH values (differences between pH before and after fermentation) ranged from 2.0 ± 0.05 to 2.3 ± 0.1 for all strains. At this temperature, the highest acidification rate obtained by the strain initially identified as N° 12, was ΔpH 2.07 and ΔTTA 62 °D, after 8 h of fermentation (Fig. 1). As expected, at 18 °C all strains showed a lower acidification rate and no change in pH values was observed until 24 h of fermentation; at this time the maximum acidification was ΔpH 2.14 and ΔTTA 72 °D produced by one strain (initially identified as N° 15).

From these results, both strains (N° 12 and N° 15) were selected for further studies. They were identified by phenotypic and genotypic techniques as *Enterococcus* (*E.*) *faecium*, rapid acidification at 37 °C (pH 4.5 at 8 h) and

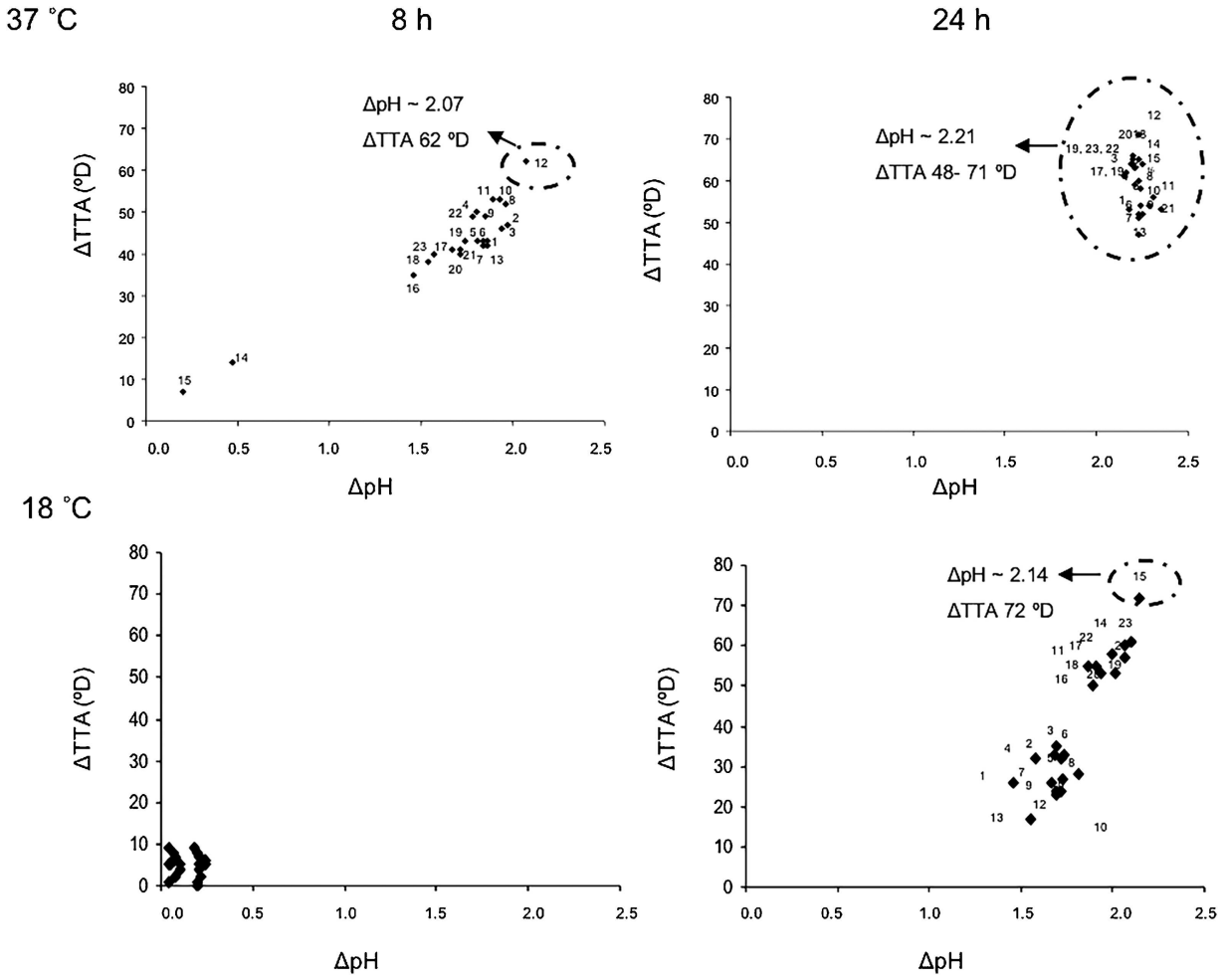

FIGURE 1 Acidifying activity (ΔpH and ΔTTA [°D]) of twenty-three strains isolated from fermented cereals during the purging stage in an artisanal tannery

Leuconostoc (*Leuc.*) *citreum* with a higher acidifying activity at 18 °C (pH 4.2 at 24 h of fermentation). Both microorganisms are deposited in the Culture Collection of CERELA under the denomination CRL 1943 (before strain N° 12) and CRL 1945 (before strain N° 15), respectively.

3.2 | Batch fermentations of selected LAB strains

Batch fermentations with each selected strain, *E. faecium* CRL 1943 and *Leuc. citreum* CRL 1945, were performed under free and controlled pH (5.5 and 6.0). The results are shown in Fig. 2. Under free pH, *E. faecium* CRL 1945 reached pH values close to 4 at 6 h of fermentation. In contrast, *Leuc. citreum* CRL 1945 reached this pH value at 12 h. These differences in acidification rate can be explained by the respective growth rates (μ); $0.66 \pm 0.03\ h^{-1}$ for *E. faecium* CRL 1945 and $0.57 \pm 0.02\ h^{-1}$ for *Leuc. citreum* and by the differences in the metabolism of these strains. In addition, the death phase of *Leuc. citreum* CRL 1945 was observed after 18 h of fermentation at free pH.

Under controlled pH, biomass production in both strains was higher than free pH (0.5 and 1.1 logarithmic units for CRL 1943 and CRL 1945, respectively). The CRL 1943 strain started the stationary phase at 6 h of fermentation, while CRL 1945 at 12 h. In this condition, a higher difference between the μ_{max} was observed; $0.65 \pm 0.02\ h^{-1}$ for *E. faecium* and $0.40 \pm 0.04\ h^{-1}$ for *Leuc. citreum.*

Residual sugars and metabolites production of both selected strains were evaluated by HPLC under the pH conditions tested. Results are shown in Table 1. Both strains consumed 100% glucose ($20\ g\ L^{-1}$) under controlled pH conditions after 24 h of fermentation, but there were found residual lactose ($4.10 \pm 0.11\ g\ L^{-1}$ for *E. faecium* and $6.85 \pm 0.09\ g\ L^{-1}$ for *Leuc. citreum*). On contrary, under free pH, residual amounts of glucose ($5.0–7.0\ g\ L^{-1}$) and galactose ($5.3–5.9\ g\ L^{-1}$) were observed in both cultures.

Lactic acid was the main fermentation product at both pH growth conditions, with better results at controlled pH: *E. faecium* CRL 1943 increased by 60% its production while *Leuc. citreum* CRL 1945 produced only 25% more lactic acid. Also, at controlled pH it was detected acetic acid ($0.30–138\ g\ L^{-1}$) and ethanol ($0.2–6.0\ L^{-1}$) in both cultures mainly CRL 1945.

3.3 | Strains compatibility

Compatibility tests between *Leuc. citreum* CRL 1945 and *E. faecium* CRL 1943 showed no clear zones around the wells, indicating that these LAB strains are compatible.

4 | DISCUSSION

LAB are key players in the production of fermented foods (yoghurt, fermented milks, cheeses, meats, wines, beers, pickles, olives, cereals) causing rapid acidification of the raw material due to production of organic acids mainly lactic acid [9]. Lactic fermentation represents a major technological process applied to cereals for human consumption [10]. Nowadays, natural fermentation of cereals is also involved in artisanal tanneries. In fact, skins are immersed in a naturally fermented cereal mixture during purging. In this study samples of fermented cereal mixture were examined in order to investigate the contribution of LAB to traditional preparations. All isolated LAB from fermented cereal mixture were cocci. These results could be due to the higher survival of cocci to adverse environmental conditions (limited monosacharides concentration, sodium chloride, low temperature), compared to lactobacilli. Gerez et al. [11] and Corsetti et al. [12] reported that cocci were also predominant in fermented wheat doughs and non-conventional flour samples.

A starter lactic culture for tannery must include strains with a fast growth rate and good acidifying activity. Furthermore, the ability to grow at low temperature must

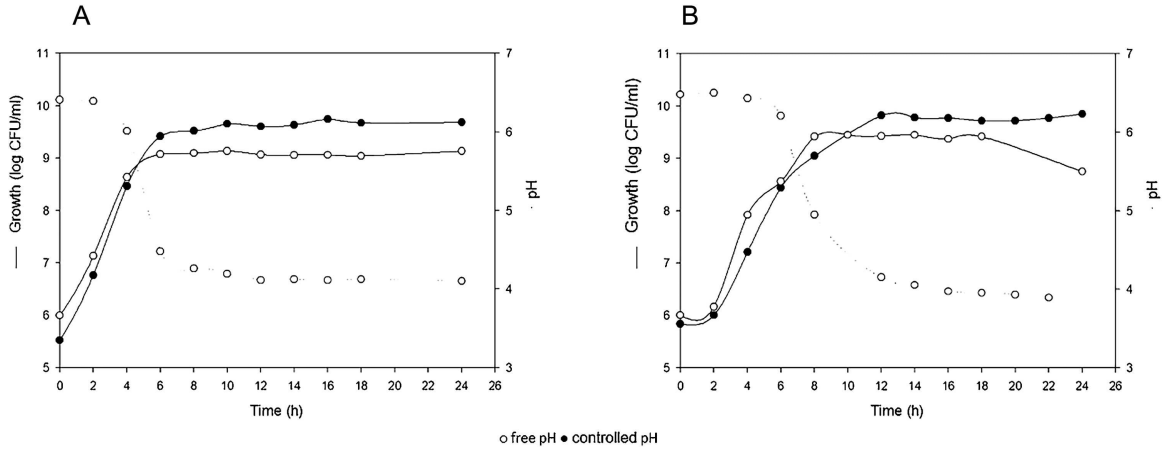

FIGURE 2 Growth measures of *Enterococcus faecium* CRL1943 (A) and *Leuconostoc citreum* CRL 1945 (B) under free and controlled pH

TABLE 1 Residual sugars and metabolite production at 24 h of fermentation under free and controlled pH for *Enterococcus faecium* CRL1943 and *Leuconostoc citreum* CRL 1945

		Sugars ($g\,L^{-1}$)			Organic Acids ($g\,L^{-1}$)		
Strain	**Fermentation conditions**	**Lactose**	**Glucose**	**Galactose**	**Lactic**	**Acetic**	**Ethanol**
CRL 1943	Free pH	5.30 ± 0.20	7.05 ± 0.08	2.15 ± 0.07	7.99 ± 0.19	0.38 ± 0.06	ND
	pH 6.0	4.10 ± 0.11	ND	ND	20.37 ± 0.21	0.93 ± 0.07	0.20 ± 0.03
CRL 1945	Free pH	5.95 ± 0.17	5.00 ± 0.12	ND	8.17 ± 0.16	1.38 ± 0.12	4.02 ± 0.10
	pH 5.5	6.85 ± 0.09	ND	ND	10.96 ± 0.11	0.30 ± 0.04	6.01 ± 0.13

ND, not detected.

be considered essential to carry out the process in cold periods. A preliminary experiment was conducted in lab-scale in order to evaluate the acidification capacity (dynamics of pH decrease and final pH) of LAB strains. Some differences in acidification properties among strains were outlined. The maximal acidifying activities were measured at 37 °C. At this temperature, most strains were able to decrease pH two units after 8 h. However, all strains with good acidification activity at 37 °C were not able to decrease the pH after 8 h at 18 °C (temperature reached during cold periods for the cereal fermentation). Acidification capability was affected by temperature as metabolic activity and growth rate decrease at low temperature [13–15]. Based on the results obtained, two strains were selected and identified as *E. faecium* CRL 1943 (rapid acidification at 37 °C) and *Leuc. citreum* CRL 1945 (high acidifying activity at 18 °C). These two genera have also been isolated from other cereal-based matrices [16–18]. LAB cocci belonging to *Leuconostoc* genera were also identified in traditional wheat sourdough from European countries [19–22]. Studies of bacterial microflora responsible for natural cereal fermentation indicate that composition depends mainly on the geographical area and the type of cereal used [21,23–25]. On the other hand, Corsetti et al. [12] observed that *E. faecium* and *E. mundtii* are the species most frequently found in fermented wheat dough. Enterococci are widely spread because of their ability to survive adverse environmental conditions. They are found as natural population of the intestine in animals [26] and, being members of the group of LAB, they play an important role in food and feed fermentations [27]. *E. faecium, E. casseliflavus*, *E. mundtii*, *E. sulfureus,* and *E. hirae*, enterococci are also isolates from vegetables, cereals, and forage plants [25]. Growth of selected microorganisms in bioreactors was studied. The scaling of the production process (10 times) resulted in a higher production of biomass at controlled pH for both strains. An important factor that affects cell growth was the pH of the media. These results are in accordance with those obtained previously for other *Enterococcus* and *Leuconostoc* strains [28–31]. Fermentation products obtained in both cases were consistent with metabolic features of each genus. Enterococci metabolize glucose to pyruvate by the glycolytic or Embden-Meyerhof-Parnas pathway (homofermentative), obtaining lactic acid as the major fermentation product under conditions of excess carbon source (glucose or lactose). When galactose is the source of carbon, heterolactic fermentation occurs, decreasing lactic acid production and producing formic, acetic acid and ethanol [32,33]. In the case of CRL 1943, a great production of lactic acid and very low levels of acetic acid and ethanol were observed, probably due to low concentrations of galactose in the culture media. Bacteria belonging to the genus *Leuconostoc* ferment glucose by the phosphocetolase pathway, being the key enzyme; final products obtained by this route are lactic acid, CO_2 and varying amounts of acetic acid and ethanol (heterofermentative pathway). For this reason, CRL 1945 produced lower amounts of lactic acid and higher concentrations of acetic acid and ethanol were observed.

In the northwest of Argentina, there are numerous artisanal tanneries; their leathers products are sold in the local market. The important role of LAB in the fermentation of cereals during the purging stage of the tanning process was demonstrated in this work. In addition, two compatible strains with high acidifying activities were selected. Also, biomass production in bioreactors was demonstrated to be very successful at controlled pH. So, these lactic acid bacteria as inoculants could represent a cost-effective innovation for family-owned artisanal businesses, as it would allow them to obtain leathers of uniform quality regardless of the climatic effects. Application of lactic inoculants in this economic area is novel, not being found in previous publication. On the other hand, acidification by lactic inoculants would make possible to avoid the use of synthetic organic acids reducing environmental pollution.

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CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interests.

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