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Screening of lactic acid bacteria for fermentation of minced wastes of Argentinean hake (*Merluccius hubbsi*)

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ABSTRACT

Sixteen strains of lactic acid bacteria were evaluated for their capacity of acidification of *Merluccius hubbsi* fish wastes obtained from a processing factory. Only three lactobacilli (*Lactobacillus buchneri* B-1837, *Lactobacillus arizonensis* B-14768 and *Lactobacillus plantarum* B-4496) were able to reduce the pH value to 4.0 or below when using glucose or sucrose as carbon source. Either with only 25 $g\,l^{-1}$ of glucose or sucrose, *L. arizonensis* B-14768 reduced the pH to 3.8 ± 0.2 within 24 h of fermentation. The acid tolerance test (pH 3.0 at 37 °C) for the strains presented D_{pH3}-values of 192, 383 and 767 min for *L. buchneri*, *L. plantarum* and *L. arizonensis*, respectively. However, at a lower pH value (pH 2.0) only *L. arizonensis* was significantly recovered after 45 min of exposure (D_{pH2} 68 min). Considering together the acidification capacity, the tolerance to other stresses (heat and bile salts) and the lower optimum temperature for the process, *L. arizonensis* is described as a suitable strain for *M. hubbsi* silage; constituting a promissory alternative for fish fermentation at location with temperate or cold climes.

Keywords: Fish silage; *Lactobacillus buchneri*; *Lactobacillus plantarum*; *Lactobacillus arizonensis*

1. Introduction

Fish processing companies generate a huge amount of under-utilized by-products. In some countries, the production of fishmeal from fish wastes is not economically viable, due to an inadequate variable supply, remote locations, and high energy and labor costs. Consequently, around 50–60% of fish wastes is usually discarded (Gildberg, 2002). Apart of the huge loss of proteins, the wastes led with an important environmental pollution (Bhaskar et al., 2011). In Patagonia (Argentina), the Argentinean hake (*Merluccius hubbsi*) constitutes a conspicuous resource for fish-export, and the amount of processing by-products demands better strategies to utilize this resource.

Fish silage can be produced using all kinds of low-value fish and fish wastes as the starter material, which is almost entirely used for animal feeding (Ganesan et al., 2009). Even though the main goal of fish silage is the utilization of the resource in an economically sustainable way, it was also demonstrated to be an effective approach to reduce or eliminate food-borne pathogens (Ganesan et al., 2009).

Early studies of fish wastes stabilization were focused on the use of inorganic acids (hydrochloric, sulphuric and, phosphoric acids) as well as organic acids (formic, propionic and, citric acids), or mixtures of them (Gildberg and Raá, 1977). On the other hand, lactic acid bacteria (LAB) constitute an interesting alternative for fish silage. Since LAB-fermented products are usually referred as healthy food, great efforts has been intended for apply them and their metabolic products in functional foods (Salminen et al., 2004; Parvez et al., 2006; Janssen et al., 2007). A requirement for these microorganisms to be used as a probiotic is their survival in acid environments during feed or food processing and storage. Even though the acid tolerance is considered an intrinsic property of LAB, significant differences in the resistance to low pH values can be found among species and particular strains (Corcoran et al., 2005). The aim of this study was the selection of LAB for silage

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of wastes of *M. hubbsi*, focused on the production of a stable secondary product for animal feeding. The tolerance to high temperatures, acidic conditions and gastrointestinal salts of the selected bacteria is also discussed.

2. Materials and methods

2.1. Materials

Minced wastes of *M. hubbsi* (trimmings, heads, frames, fins, skin, and viscera) were purchased immediately after processing from an industrial plant located in Rawson, Chubut, Patagonia (Argentina). The samples were transported and, kept at 4 °C and processed within 12 h.

2.2. Microbial sources

LAB strains (*Lactococcus lactis* subsp. cremoris B-634, *Lactobacillus fermentum* B-1840, *Lactobacillus sakei* B-1917, *Lactobacillus casei* subsp. casei B-1922, *Carnobacterium divergens* B-14830, *Lactobacillus plantarum* 379, *Lactobacillus buchneri* B-1837, *Lactobacillus brevis* B-4527, *Lactobacillus breve* 381, *Lactobacillus casei* 382, *Lactobacillus fermentum* 383, *Lactobacillus helveticus* 384, *L. plantarum* B-4496, *Lactobacillus arizonensis* B-14768 and *Carnobacterium maltaromaticum* B-14852 were provided by the United States Department of Agriculture (USDA) collection. *Lactobacillus* sp. SES380 strain was isolated at the Chemistry Department (UNLPam).

2.3. Screening of LAB

Inoculums were obtained by overnight cultivation of LAB in Man Rogosa Sharpe (MRS) medium containing ($g\,l^{-1}$): 10 enzymatic digest of animal tissue; 10 meat extract; 5 yeast extract; 20 dextrose; 2 di-potassium hydrogen phosphate; 1 Tween® 80; 2 di-ammonium hydrogen citrate; 5 sodium acetate; 0.1 magnesium sulfate; 0.05 manganese sulfate; final pH 6.5 ± 0.2 at 25 °C (MRS, Biokar Diagnostics, France). For fermentation, flasks of 0.12 l capacity containing 10 g minced fish were supplemented with 10 ml of glucose or sucrose solutions, to render final concentrations between 25 and 75 $g\,l^{-1}$. The 16 strains were inoculated (25 $ml\,l^{-1}$) in the flasks previously sterilized in autoclave (15 min – 121 °C), and were incubated at 28 or 37 °C for 96 h. Debris, composed by fishbone and fish fins, were removed from the homogeneous suspension. The experiments were performed three times and each reading represents the average with the respective standard deviation. The pH decay during fermentation was fitted to the following exponential equation:

$$pH = pH_{final} + (pH_{initial} - pH_{final}) \times 2^{-t/t_{1/2}} \quad (1)$$

where pH_{final} is the minimum pH reached, t the time, $t_{1/2}$ is the time required for one half of the total change of pH.

2.4. Stirred reactor (SR)

A 12-l glass fermenter was built up for the procedure. The fermenter was fixed in a water bath for temperature control (29 ± 1 °C). Sampling, inoculation, and gas flow out ports were performed with polyamide pipe, the last connected to a filter of 0.2 μm pore size (Whatman filters, UK). The ten liters working volume was composed of mince hake (7.0 kg) and 3.0 l of sucrose solution rendering a final concentration of 25 $g\,l^{-1}$. Other carbon sources assayed were 30 $g\,l^{-1}$ of grounded seeds of wheat, sorghum, barley, sunflower and corn. The medium was sterilized at 121 °C for 2 h. The fermenter was placed on a magnetic stirring plate and was constantly stirred at 150 rpm. The SR was inoculated with 250 ml of a 24 h old culture of *L. arizonensis* performed in MRS medium.

2.5. Tolerance of Lactobacilli against acid conditions, heat and bile salts exposure

Fish silages obtained from *L. plantarum* B-4496, *L. arizonensis* B-14768 and *L. buchneri* B-1837 strains were centrifuged at 3000 rpm for 15 min and washed twice with 100 mM sterile phosphate-buffered saline (PBS) (pH 7.0). The cell suspension (0.1 ml) was added to 5 ml of PBS at pH 1–7 acidified with hydrochloric acid, and incubated at 37 °C. For bile salts tolerance, 5 ml of MRS cultures containing 20 $g\,l^{-1}$ Oxgall were performed at 28 or 37 °C during 48 h; and for heat tolerance, the cell suspension was incubated 1 h at 60 °C. The survival was monitored by measuring colony forming units (CFU ml^{-1}) in MRS agar (48 h, 37 °C) according to Kacem and Karam (2006). The experiments were performed by triplicate and the resistance value (death kinetics) of LAB strains was characterized in terms of decimal reduction time, *D*-value, which is the exposure time required, under specified set of conditions, to cause one $\log_{10}$ or 90% reduction of the initial population (N_0). The *D*-value was determined from the inactivation kinetic curve fitted to the equation:

$$N = N_0 \times 10^{-t/D} \quad (2)$$

where N is the surviving population (CFU ml^{-1}) at each exposure time, N_0 is the initial bacterial population (CFU ml^{-1}), and t is the exposure time (min). For acidic conditions (pH 3) and bile salt exposure, the method experienced problems fitting curves to experimental data showing pronounced asymptotic tails (<10% CFU ml^{-1}). The tails were restricted in the regression analysis by including only two points after the establishment of the tail to improve the fitting according to Klotz et al. (2007).

2.6. Analytical assays

The moisture content was determined by air drying (method AOAC 950.46). The fat content was assessed after 16 h extraction with ethyl ether (AOAC 948.16), total titratable acidity (AOAC 947.05), and crude protein contents was determined by Kjeldahl method (N × 6.25) (AOAC 970.42) (AOAC, 1990). Fatty acid composition was determined by gas chromatography with a flame ionization detector (Hewlett Packard Model 6890) and shown in the text as percentages of the fat content. Calcium and phosphorous were determined by inductively coupled plasma, atomic emission spectrometry (ICP-AES).

2.7. Statistical analysis

The experiments followed a Complete Randomized Design (CRD). The obtained data were subjected to analysis of variance (ANOVA). Multiple Range Test of Duncan was used to compare between means of treatment at 5% probability.

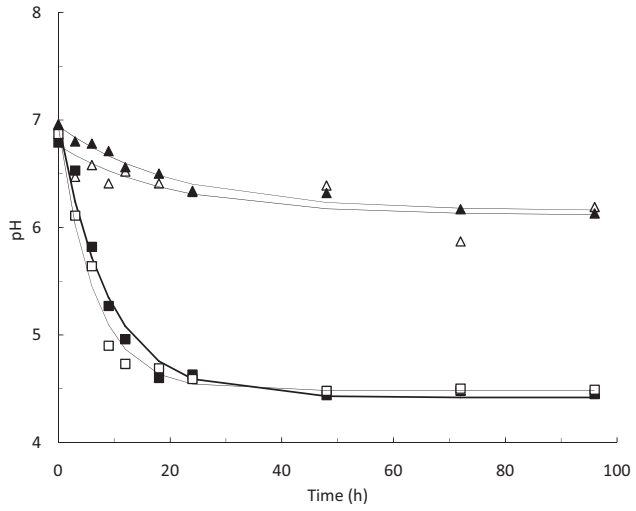

Fig. 1 – Acidification profile of *L. lactis* subs. *cremoris* B-634 using 2 different carbon sources (sucrose or glucose) during fermentation of minced *M. hubbsi*. Each value represents the means of three ± SD: (■) 2.5% (w/v) glucose, (□) 5.0% (w/v) glucose, (▲) 2.5% (w/v) sucrose, (Δ) 5.0% (w/v) sucrose.

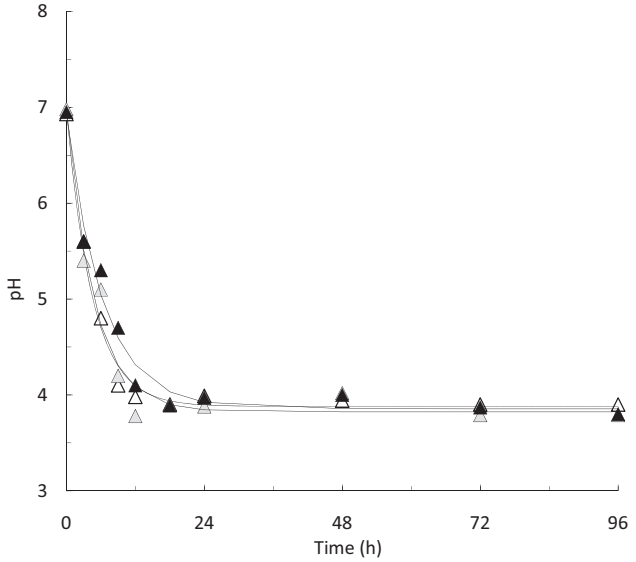

Fig. 2 – Acidification profile of *M. hubbsi* by-products during *L. arizonensis* B-14768 fermentation with different concentrations of sucrose: (Δ) 2.5% (w/v), (△) 5.0% (w/v), (▲) 7.5%. Each value represents the means of three ± SD.

3. Results and discussion

3.1. *Fermentation of processing by-products of M. hubbsi with lactic acid bacteria*

The hake based medium had an initial pH between 6.5 and 7.5. In order to avoid fish rotting the material must be acidified to pH <4.5 (Dapkevicius et al., 2000), and the acidification rate is usually considered a key variable of the process. Since hake waste is a poor source of fermentable sugars, an extra energy source must be added for bacterial growth, as it was previously reported for other fish fermentations (Shirai et al., 2001). Sixteen strains of lactic acid bacteria were screened for the fermentation of hake wastes in presence of different concentrations (25, 50 or 75 g l^{-1}) of glucose and sucrose for an efficient production of lactic acid (Gildberg, 2002). The hake wastes fermented without previous treatment generated, gradients of pH and production of H_2S (data not shown), consequently we proceed to homogenize the samples before fermentation. Among them, six strains (*L. lactis* subsp. *cremoris* B-634, *L. fermentum* B-1840, *L. sakei* B-1917, *L. casei* subsp. *casei* B-1922, *C. divergens* B-14830 and *L. plantarum* 379) showed a differential acidification kinetic according to the carbon source used. In presence of glucose, they were able to reduce the pH value to 4 after 24 h, whereas with sucrose the pH remained between 6 and 7 during the entire process. As an example, the differential profile in the acidification rate for the strain *L. lactis* subsp. *cremoris* B-634 is shown in Fig. 1. The *L. fermentum* B-1840 and *L. casei* subsp. *casei* B-1922 reached an average pH 4.1 ± 0.3 with higher glucose concentrations (50–75 g l^{-1}) in 24 h fermentation. But these strains were not able to acidify the broths neither with lower concentration of glucose nor with sucrose at any concentration; similarly as it is mentioned above for *L. lactis* subsp. *cremoris* B-634 (Fig. 1).

A second group constituted by the other ten strains (*L. buchneri* B-1837, *L. brevis* B-4527, *L. breve* 381, *L. casei* 382, *L. fermentum* 383, *L. helveticus* 384, *L. plantarum* B-4496, *L. arizonensis* B-14768, *C. maltaromaticum* B-14852 and *Lactobacillus* spp. SES380) did not present important differences in the acidification rate between the supplied carbon sources. Nevertheless, only three strains reduced the pH to values as low as 4 with both carbon sources (Table 1). Although both sugars could be used for fish fermentation, sucrose is preferable because of the lower costs and higher availability. The homofermentative bacteria *L. arizonensis*, isolated for the first time from Jojoba meal 10 years ago, showed the most efficient reduction of pH for *M. hubbsi* fermentation for both carbon sources among the assayed LAB (Swezey et al., 2000). Fig. 2 shows the acification profile of L. arizonensis with different concentration of sucrose.

The time required for one half of the total change of pH ($t_{1/2}$) was estimated from Eq. (1) for *L. arizonensis* B-14768. The variable $t_{1/2}$ is a parameter related to the acidification rate in the conditions of the assay. The $t_{1/2}$ showed a tendency to diminish – e.g., higher acidification rate – at low concentrations of sucrose (Fig. 3). Therefore, the observed tendency

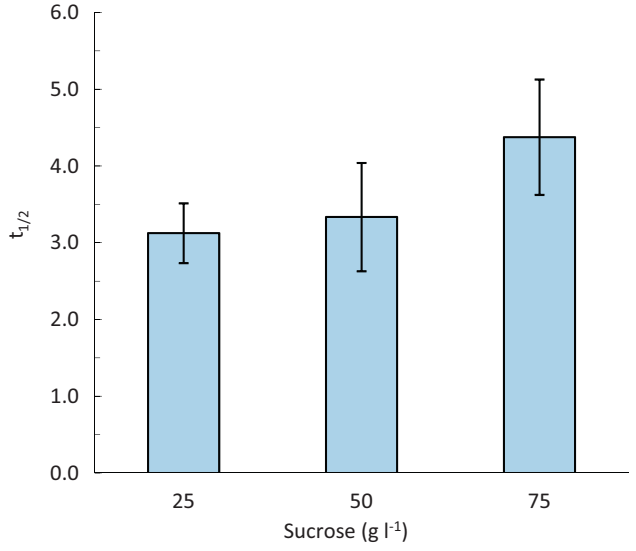

Fig. 3 – Effect of carbon source concentration on the acidification rate ($t_{1/2}$) of *M. hubbsi* fermentation by means of *L. arizonensis* B-14768. Each value represents the means of three ± SD.

Table 1 – Acidification of Argentinean hake wastes by selected *Lactobacillus* strains.

	Temperature	Final pH	Minimum sucrose concentration required to reach pH 4	Time
L. plantarum B-4496	37 °C	3.90 ± 0.05	25 g l^{-1}	72 h
L. arizonensis B-14768	28 °C	3.99 ± 0.08	25 g l^{-1}	24 h
L. buchneri B-1837	37 °C	4.08 ± 0.02	75 g l^{-1}	72 h

Table 2 – Survival of *Lactobacilli* after exposure to acid pH, bile salts (2%, w/v Oxgall) and high temperature (60 °C).

	D (min)				
	60 °C	pH 3	pH 2	pH 1	2% Oxgall
L. plantarum B-4496	32	383	14	<10	1150
L. arizoniensis B-14768	209	767	68	<10	329
L. buchneri B-1837	164	192	10	<10	230

indicated that 25 g l^{-1} would be the concentration of choice to scale up the process. Interestingly, the initial amount of carbon source added has been reported to be a critical factor for lactic acid fermentation. Shirai et al. (2001) reported that fermented shrimp wastes supplemented with glucose concentration below 75 g l^{-1} were not able to reach pH values lower than 5.6. Usually, 100 g l^{-1} or higher concentrations are needed for an efficient acidification (Cira et al., 2002 and references in it). Although we obtained a similar fermentative behavior with *L. buchneri* B-1837, increasing the efficiency at high carbon source concentrations, the strains *L. plantarum* B-4496 and *L. arizonensis* B-14768 showed the same performance with one third of the sucrose concentration (25 g l^{-1}). Remarkably, *L. arizonensis* B-14768 reached the desired pH after 24 h fermentation, in comparison with the 72 h required for the other two strains (Table 1). These three strains were selected for further studies of tolerance to different stresses to test their probiotic potential and the resistance at pelleting temperatures (60 °C).

3.2. *Tolerance of LAB at different stress conditions (acidic environment, bile salts and heat)*

The heat inactivation under isothermal conditions at 60 °C, and exposure to acidic conditions and bile salts were found to follow first order kinetics. Experimental results were analyzed using a one step non-linear regression method, calculating the decimal reduction times using Eq. (2).

Pelleting of the fermented fish is required to develop a stable product for animal feeding. In order to retain the probiotic activity in the product, the cell should stay alive after the pelleting process. For that reason, the heat resistance of the selected LAB was determined from the reduction of the number of viable cells (colony forming units, CFU) after heating the cell suspension at 60 °C. *L. plantarum* B-4496 was the more sensitive strain with a decimal reduction time of 32 min, in comparison with the other strains that presented *D*-values between 2.7 and 3.4 h (Table 2). Some thermophylic strains of LAB were reported to tolerate high temperatures (60–80 °C) for 5–10 min (Salminen et al., 2004; Christiansen et al., 2006; Golod et al., 2009), in this work significant amount of 2 strains of LAB were recovered after the thermal treatment at 60 °C, indicating the potential of these strains for processes that involve high temperatures like pelleting, cooking or spraying.

To exert their probiotic effect, LAB strains not only have to survive acid environments during food processing but also during the first 20–30 min after meal, where the pH values in the gut are even lower (Marteau et al., 1997; Corcoran et al., 2005). The cell suspensions were incubated at 37 °C during 3 h at pH 3, 2 and 1 (Table 2). At pH 3.0, the three strains showed important recoveries, being *L. plantarum* B-4496 and *L. arizonensis* B-14768 the more resistant strains with a *D*-values of 6 and 12 h, respectively. Although pH 1.0 was deleterious for all the strains (*D*-values <10 min), at pH 2.0 *L. arizonensis* was the only strain recovered in significant amounts after 45 min of exposure. These results are in agreement with other reports that show an important acid resistance for *L. plantarum* B-4496 (Kacem and Karam, 2006; Sheehan et al., 2007). Nevertheless, the acid tolerance of *L. arizonensis* B-14768 would be of practical importance to reach the intestine in sufficiently high number of living cells (Dunne et al., 2001; Marteau et al., 2001).

The bile salt tolerance was assessed as indicative parameter for the potential of a given strain to act as probiotic. Resistance to bile salts varies among the lactic acid bacteria species and also among strains (Havenaar et al., 1992; Xanthopoulos et al., 1997). The level of bile salts in animals or human gut is approximately 3 g l^{-1}, and it rises to higher concentrations during the first hour of digestion (Fernández et al., 2003). Bile salt tolerance was evaluated using 20 g l^{-1} Oxgall in the cell suspensions at 37 °C. In all cases Oxgall was deleterious for the selected LAB but the resistances were highly different among strains. *L. plantarum* B-4496 was the more resistant with *D*-value of 19 h, while *L. buchneri* B-1837 and *L. arizonensis* B-14768 were poorly detected after 4 h and 6 h, respectively.

3.3. *Scale up fish fermentation in a stirred reactor*

The fish fermentation was scaled up in a 12-l bench fermentor based on the performed optimization: the microorganism of choice was *L. arizonensis* B-14768 and sucrose level was set at the minimum concentration required for acidification (25 g l^{-1}) (Fig. 4). Grounded cereals 30 g l^{-1} (wheat, sorghum, barley, sunflower seeds, and corn) were also assayed as alternative carbon sources, but using these carbon sources the pH values did not decreased more than 4.4–5.1 after 24 h fermentation.

The fish wastes fermentation using a working volume of 10 l with 25 g l^{-1} sucrose was monitored during 2 days. The final product presented a high protein content of 63.5% (w/w) of dry weight, while the fat content was reduced from 13.4 g l^{-1} of the starting material to a range between 5 and 7 g l^{-1} in the final product. The main fatty acids did not vary irrespective of the starting material, being the abundant ones, palmitic (35.3%), oleic (34.8%) and palmitoleic (10.7%) acids. The soluble phase contained 64.3 mg kg^{-1} calcium and 2.8 g kg^{-1} phosphorous, while lactic acid production increased up to 19.7 g l^{-1} at the end of fermentation (48 h). The bacterial concentration rose up from 4.5×10^5 till 8.5×10^6 CFU ml^{-1} (Fig. 4). The final product reached a pH value of 3.8 ± 0.3 after 48 h, comparable to those obtained for freshwater carps fermentation using 75 g l^{-1} molasses as carbon source (Ganesan et al., 2009) and

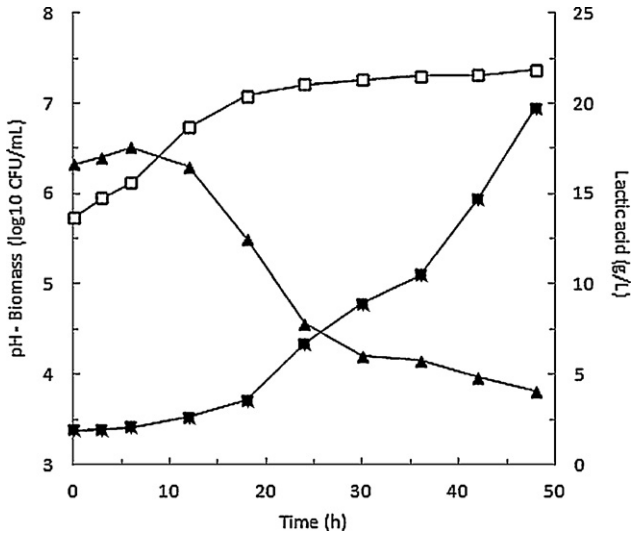

Fig. 4 – *M. hubbsi* fermentation using *L. arizonensis* B-14768 in a bench scale fermentor (10 l working volume). The medium contained 2.5% (w/v) sucrose as carbon source. Each value represents the means of three ± SD: (■) lactic acid concentration ($g\,l^{-1}$), (▲) pH, (□) biomass (log_{10} CFU ml^{-1}).

significantly lower than the obtained for shrimp fermentation (pH 4.9) using 10 $g\,l^{-1}$ sucrose (Cira et al., 2002).

4. Conclusion

Several LAB were evaluated for fermentation of processing wastes of *M. hubbsi* and three were selected as the most promissory strains (*L. plantarum* B-4496, *L. buchneri* B-1837 and *L. arizonensis* B-14768). These microorganisms reduced the pH value to around four by adding fermentable substrates such as glucose or sucrose. The versatility of *L. arizonensis* for both carbon sources, its lower optimal growth temperature (28 °C) and its capacity to grow at even lower temperatures (10 °C), makes this strain a promissory alternative for uncontrolled temperature fish fermentation in temperate to cold climes.

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