Lactobacillus plantarum strains isolated from naturally fermented sausages and their technological properties for application as starter cultures

Lactobacillus plantarum isolados de salames artesanais naturalmente fermentados e suas propriedades tecnológicas como culturas iniciadoras

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Resumo

No presente estudo foram investigadas as propriedades tecnológicas de culturas de *L. plantarum*, isoladas de salames artesanais, naturalmente fermentados, manufaturados na região Sul do Brasil, a fim de obter um cultivo iniciador. As propriedades tecnológicas investigadas foram as seguintes: habilidade das culturas para crescer em diferentes valores de pH, em diferentes concentrações de sal e na presença de sal de cura comercial; rápida produção de ácido, produção do isômero D – ou L – ácido láctico, atividade nitrato redutase, atividade antagonística e estabilidade das culturas após processo de fermentação, concentração e liofilização. Todas as culturas apresentaram eficiência quanto às propriedades tecnológicas investigadas.

Palavras-chave: *L. plantarum; propriedades tecnológicas; salame.*

Abstract

In the present study, technological properties of *L. plantarum* strains isolated from naturally fermented sausages manufactured in the South region of Brazil were investigated in order to obtain a starter culture. The technological properties evaluated were the following: ability to growth at different pH values, at different temperatures, in different salt concentrations and in the presence of commercial curing salt, fast production of acid, determination of D – and L – lactic acid; nitrate reductase activity; antagonistic activity and stability of the isolated cultures after fermentation, concentration, and freeze-drying process. The isolated strains showed effectiveness to improve technological properties as starter cultures.

Keywords: *L. plantarum; technological properties; sausage.*

1 Introduction

The manufacture of fermented foods has a long tradition. At first, there was a purely empirical principle without the connection between metabolic activity of microorganisms (so-called "house flora") and desired changes in the product (GEISEN; LÜCKE; KRÖCKEL, 1992). The fermentation process was used to improve shelf-life and safety of foods enabling people in moderate and cold regions to survive winter seasons and drought periods (HOLZAPFEL, 1997). Spontaneous fermentation of sausages is characterized by the participation of lactic acid bacteria, Gram-positive, catalase-positive cocci, yeasts, and moulds (BUCKENHÜSKES, 1993).

Modern starter cultures are selected either as single or multiple strains specifically due to their adaptation to the substrate or raw material (HOLZAPFEL, 2002). The Iinoculation of sausage batter with a starter culture composed of selected lactic acid bacteria, i. e. homofermentative lactobacilli and/or pediococci and Gram-positive, and catalase-positive cocci (staphylococci and/or kokuriae) improves quality, safety, properties standardization, including flavor and color, and shortening in the ripening time (LEROY; VERLUYTEN; VUYST, 2006; RANTSIOU et al., 2005). Lactic acid bacteria have the main role in this microbial consortium since they affect both the technological properties and the microbial stability of the final product through the production of lactic and acetic acids and the consequent pH decrease (DROSINOS et al., 2007).

Meat fermentation by natural lactic acid bacteria can sometimes fail leading to products of poor quality. For this reason, the addition of starter cultures has been recommended and has become common in the manufacture of several types of fermented sausages (ANDRIGUETTO; ZAMPESE; LOMBARDI, 2001; HOLZAPFEL, 2002). In artisanal production of traditional fermented sausages, it is important to use starter cultures consisting of lactobacilli isolated from local products and that are well adapted to the particular product and to the specific production technology (ANDRIGUETTO; ZAMPESE; LOMBARDI, 2001). Starter cultures contain lactic acid bacteria

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originated from meat and are considered well adapted to the ecology of meat fermentation (HUGAS et al., 1993). The fitness of commercial meat starter cultures when applied to a particular type of salami is questionable since a culture that performs well in one type of fermented sausage is not necessarily efficient in another type (LEROY; VERLUYTEN; VUYST, 2006).

The aim of the present work was to investigate technological properties of *Lactobacillus plantarum* strains isolated from naturally fermented sausages manufactured in the South region of Brazil in order to obtain a starter culture for fermented meat products.

2 Materials and methods

2.1 Bacterial strains and growth conditions

Seven strains of *L. plantarum* were used in this study (AJ2, AL2, R2, AF5, AD3, N3, and AM2). These strains were selected from a previous study of morphological, phenotypic, and molecular characterization of ten strains isolated and characterized by Sawitzki et al. (2007).

All strains were grown in de Man Rogosa and Sharp agar (MRS; Merck, Darmstadt, Germany) plates and were anaerobically incubated at 37 °C for 48 hours. They were sub-cultured twice (1% *inoculum*, 37 °C/24 hours) in 10 mL MRS broth (Merck, Darmstadt, Germany) and kept frozen at 80 °C in the presence of 20% glycerol.

To test the antagonistic activity, the following reference strains were acquired from the *Collection André Tosello Foundation*: *Staphylococcus xylosus* ATCC 29971, *Listeria monocytogenes* NTC 098630, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 12598. One strain of *Staphylococcus xylosus* isolated from an artisanal sausage and manufactured without the addition of a starter culture was also used for the tests of antagonistic activity.

2.2 Technological properties

Technological properties for each natural strain of *L. plantarum* were investigated according to Buckenhüskes (1993), Holzapfel (2002), Lee, Kim, Kunz (2006), Saarela et al. (2006). In addition, the growth of the strains in the presence of commercial curing salt was also evaluated according to the manufacturer's instructions.

In order to test the antagonistic activity, strains which present poisoning risks in fermented sausage were selected (LEROY; VERLUYTEN; VUYST, 2006). *S. xylosus* strains were also tested once it is one of the most important microorganisms used as starter culture (GEISEN; LÜCKE; KRÖCKEL, 1992).

All tests were conducted in duplicate.

Ability to grow at pH 3.9, fast production of acid, and determination of D–/L– lactic acid

Growth at pH 3.9 was observed after 3 days of incubation at 37 °C on MRS agar (Merck, Darmstadt, Germany) plates adjusted with HCl (1M). The fast production of acid was monitored by measuring the pH decrease over a 12 hour-fermentation (MRS broth, Merck, Darmstadt, Germany). Fermentation was carried out in a fermentor (New Brunswick scientific model Bioflo 2000, New Brunswick, USA). Each culture of *L. plantarum* was first growth in 45 mL of MRS broth (Merck, Darmstadt, Germany) for 12 hours at 37 °C when the inoculum was added in 4.5 L of the fermentation medium. The lactic acid isomer produced by each strain was determined with a D–/L– lactic acid enzymatic kit (R – Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions.

Growth in high salt concentration and in the presence of commercial curing salt

Growth in high salt concentration was observed after 3 days of incubation at 37 °C on MRS agar (Merck, Darmstadt, Germany) plates added with 6.0 and 7.0% of NaCl (Merck, Darmstadt, Germany), respectively. Growth in the presence of commercial curing salt was observed after 3 days of incubation at 37 °C on MRS agar (Merck, Darmstadt, Germany) plates added with commercial curing salt (*Cura 102 – Duas Rodas Industrial Ltda, Jaraguá do Sul, Brazil*), with sodium nitrate and sodium nitrite in respective concentrations of 300 and 150 $mg.kg^{-1}$.

Nitrate reductase activity

For the nitrate reductase test, a swab of each culture of *L. plantarum* was grown on MRS agar (Merck, Darmstadt, Germany) plates (anaerobically incubated at 37 °C for 48 hours) and suspended in sterile peptone water 0.1% with turbidity equivalent to 0.5 McFarland. A 1.0 mL aliquot of homogenized bacterial suspension was added to a sterile tube containing nitrate broth (DIFCO, Lawrence, USA). All tubes were incubated anaerobically at 37 °C for 48 hours. After the incubation period, 1 drop of each reagent of the NIT test (NIT 1 + NIT 2 reagents bioMérieux®sa, Marcy l'Etoile, France) was added to each tube. After 10 minutes, the presence of red color indicated positive reaction to the reduction of nitrate to nitrite. A negative control with no substrate and a positive control with a culture of *S. xylosus* positive for nitrate redutase were used.

Stability of cultures after fermentation, concentration, and freeze-drying process

Cells (45 mL of an initial inoculum – 10^9 $cfu.mL^{-1}$) were grown in 4.5L of MRS broth (Merck, Darmstadt, Germany) using a fermentor (New Brunswick Scientific model Bioflo 2000, Edison, USA) under the following conditions: temperature of 37 °C, stirring at 80 rpm, and aeration of 0.7 vvm (L filtered air atmospheric/L medium/minutes) for 12 hours. The bacterial population was determined during fermentation by plate count analysis and respective optic density at 600nm of the fermentation medium. After 12 hours of fermentation (DO 2.408 and respective microbial population of Log 9 $cfu.mL^{-1}$ – logarithmic growth phase) cells were concentrated by centrifugation at 4000 × g, 30 minutes, 4 °C (Novatécnica RC, model NT825, Piracicaba, Brazil), re-suspended to 1/50 of the original broth volume in reconstituted skimmed milk (10% $w.v^{-1}$) as cryoprotectant, and stored at – 20 °C. Freeze-drying was performed in a freeze-dryer (Terroni-Fauvel model LT 1000/8, São Carlos, Brazil) for

24 hours. Freeze-dried cultures were stored at – 20 °C. Freeze-dried cultures were re-suspended in sterile 0.1% peptone water (2.5×10^{-3} g.mL^{-1}) after 4 weeks and 6 months of storage and inoculated into MRS agar using the pour plate technique (Merck, Darmstadt, Germany) (anaerobically at 37 °C for 48 hours). The bacterial population was determined by plate count analysis in order to evaluate the stability of the cultures at –20 °C.

Antagonistic activity

The antagonistic activity of isolated cultures of lactic acid bacteria was detected by the spot-on-lawn method according to Lewus, Kaiser, Montville (1991), Okereke, Montville (1991). TSA agar (Tryptic Soy Agar; Merck, Darmstadt, Germany) supplemented with 0.5% yeast extract (Merck, Darmstadt, Germany) was named TSAYE and used as the bottom (bacteriocin production) agar. Two microliters of each overnight MRS broth cultures of *L. plantarum* were spotted onto TSAYE plates and incubated anaerobically at 37 °C for 48 hours. After incubation, an overlay with approximately 8 mL of BHI (Brain Heart Infusion; Merck, Darmstadt, Germany) containing 1% agar and 10^5 to 10^6 cfu.mL^{-1} of each test culture was added to each plate. The cultures tested were the following: *S. xylosus* ATCC 29971, *L. monocytogenes* NTC 098630, *E. coli* ATCC 25922, *S. aureus* ATCC 12598, and *S. xylosus* isolated from artisanal sausage. Plates with the overlay and a control plate (without any tested culture) were incubated anaerobically at 30 °C for 48 hours and observed regarding to inhibition zones. The antagonistic activity was positive when the width of the clear zone (halo) around the colonies of the producing strain was 3.0 mm or larger according to Sarkar, Banerjee (1996).

3 Results and discussion

All *L. plantarum* isolated strains were able to grow at pH 3.9 at 37 °C on MRS agar. During fermentation in MRS broth, they presented fast production of acid since the average pH values (of the respective fermentation medium) decreased from 6.48 to 4.43 after 16 hours of fermentation (Figure 1).

The most important change brought about by lactic acid bacteria in a ripened meat product is the decrease of pH (to below 5.0) by the secretion of lactic acid (GEISEN; LÜCKE; KRÖCKEL, 1992). The ability of strains to produce acid and growth at pH 3.9 is significant once the acid production and consequent decrease of pH in sausages can cause coagulation of meat proteins, necessary reactions for color formation, and improvement of the product stability (BUCKENHÜSKES, 1993). Due to the decrease of the water-binding capacity of meat proteins, the acidification accelerates drying out and thus shortens the processing time (JESSEN, 1995).

The ability of lactic acid bacteria, in particular lactobacilli, to decrease pH prevents the growth of pathogenic and spoilage microorganisms improving the hygienic safety and storage of meat products (LUCKE, 1985; SAMELIS et al., 1994). A rapid pH drop to below 5.3 proved to be important for the inhibition of salmonella and *S. aureus* if products since such products are fermented at temperatures above 18 °C (SCHILLINGER; LÜCKE, 1989). The growth of *Clostridium botulinum* and *Clostridium sporogenes* were susceptible to nitrite inhibition only at pH values below 7.0 (GRAY; PEARSON, 1984). The acidification of ground meat during the production of dry sausage, for instance, can be obtained by adding gluconа-delta-lactona or by fermentation. In both cases, the shelf life safety and slice ability of the sausage will be achieved, but the original taste and flavor can only be obtained by fermentation (BUCKENHÜSKES, 1993).

Lactic acid is produced by lactic acid bacteria in two isomeric forms: L – lactic acid and D – lactic acid (SHU; HÅKANSON; MATTIASSON, 1995). The isomers L – and D –, produced during fermentation, are typically related to the *Lactobacillus* genus from which they are produced: for example, D (–) lactic acid from *L. delbrueckii* (all subspecies), L (+) from *L. case*, and a racemate (DL) from *L. sakei* and all heterofermentative lactobacilli (HOLZAPFEL, 2002). *L. plantarum* produces a racemate DL – lactate (KLANDER; WEISS, 1986). Most lactobacilli are DL – lactate producers, but the ratio of the two isomers is slightly variable (GOFFIN et al., 2005).

In the present work, all the *L. plantarum* strains isolated produced DL – lactate on the average ratio of 67.23% of L – lactic acid and 32.76% of D – lactic acid (w.v^{-1}) as a final product of sugar fermentation. *L. plantarum* strain ATCC 8014 produced 1.16% of D – lactic acid and 98.84% of L – lactic acid (w.v^{-1}) (Table 1). Lactic acid isomers showed differences in antibacterial mechanisms, for example, *L. monocytogenes* (relatively acid tolerant pathogen) is more sensitive to D – than to L – lactic acid (GRAVESEN et al., 2004). In this case, the production of D – lactic acid by *L. plantarum* isolated is an important factor, but further investigation of the concentration of this isomer in fermented foods and beverages is necessary, once high levels of the D – lactic acid are not hydrolysed by D – lactic dehydrogenases in humans being capable of causing acidosis (LIU, 2003; HOLZAPFEL, 2002; ZHANG et al., 2003).

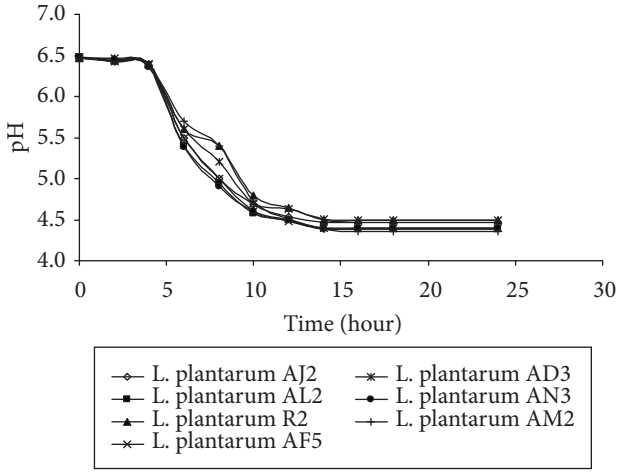

Figure 1. pH values in the fermentation medium (de Man Rogosa and Sharp broth), inoculated with isolated *L. plantarum* strains over 24 hours of fermentation.

Tolerance to NaCl is another significant feature for choosing a strain as starter culture in dried fermented products (ROVIRA et al., 1997). Strains that are able to grow at 6.5% NaCl might result from a selection of resistant strains to high salt concentration during sausage processing NaCl – sensitive strains are present at the beginning of the process and may stop growing when NaCl concentration becomes too high (AMMOR et al., 2005a). According to Olesen and Meyer, Stahnke (2004), the initial NaCl concentration (3.0%) in the sausage is a normal one. However, the final NaCl concentration after the maturation of the sausage can increase to 4.2-6.0% due to the loss of moisture in the product (MORETTI et al., 2004; PAPADIMA et al., 1999; ZANARDI et al., 2004).

Papamanoli et al. (2003) found that seven *L. plantarum* strains isolated from naturally fermented dry sausage were able to grow in 6.5, 8.0, and 10% (w/w) of NaCl. In the present study, 3 out of 7 isolated strains (*L. plantarum* AL2, *L. plantarum* AD3 and *L. plantarum* AM2) were able to grow on MRS agar supplemented with 6.0 and 7.0% of NaCl.

Curing salt as well as lactic acid and NaCl are important elements concerning sensory, microbial and physiochemical characteristics of meat products. In this study, all isolated strains were able to grow on MRS agar plates supplemented with commercial curing salt in the respective concentration for fermented sausages (300 mg.kg^{-1} of nitrate and 150 mg.kg^{-1} of nitrite).

According to Deibel (1974); Smith, Palumbo (1983), salt and nitrite-tolerant (growth vigorously at 6% NaCl and 100 ppm nitrite) are desirable characteristics of a meat starter culture. Tolerance to cure salt is another significant factor for selecting a strain as starter culture in dried fermented meat products because the curing is an efficient conservation technique. Nitrite as curing agent is a preservative and the only agent with protective effect against toxin-forming bacteria such as *C. botulinum* (CAMMACK et al., 1999). According to Gray, Pearson (1984), citing Kramlich, Pearson, Tauber (1973), nitrite has several important functions in meat products: (1) stabilize color, (2) contribute to the characteristic flavor of cured meat, (3) inhibit the growth of a number of food poisoning and spoilage bacteria, especially of *C. botulinum*, and (4) retard the development of rancidity. The use of nitrate is interesting because it improves flavor when compared to nitrite (WIRTH, 1991). According to Gray, Pearson (1984), the American Meat Institute stated that nitrate is changed to nitrite by the action of microorganisms and these organisms may play an important role on the typical flavor produced in meat products. For Marco, Navarro, Flores (2006), the addition of nitrate and/or nitrite in the manufacturing of dry-fermented sausage hardly affects the sensory characteristics. However, nitrite and nitrate affect both the oxidative process and the generation of volatile compounds originated from the growth and metabolism of microorganisms.

In a long curing process, nitrate is necessary as a source of nitrite by the action of nitrate reductase enzymes (TOLDRÁ, 2005 mentioned by MARCO; NAVARRO; FLORES, 2006). To enable the reddening process of the cured meat, the reduction of nitrate to nitrite is essential. Under conditions of sausage fermentation, this reaction is only possible by a nitrate reductase, which normally derives from Micrococcacea (BUCKENHÜSKES, 1993). However, some lactobacilli strains may reduce nitrate (NO_3^-) to nitrite (NO_2^-) and monoxide nitrogen (NO) under anaerobic conditions (WOLF; HAMMES, 1988). Four *Lactobacllus fermentum* strains tested by Xu, Verstraete (2001) showed nitrate redutase activity under anaerobic conditions, but two *L. plantarum* strains did not show the respective activity. Some strains of *L. plantarum* are able to reduce nitrate under low glucose concentration and pH at 6.0 or higher (KLANDER; WEISS, 1986). In the present study no *L. plantarum* isolated showed nitrate reductase activity in the nitrate broth incubated anaerobically at 37 °C for 48 hours. According to BUCKENHÜSKES (1993), the curing of meat products in the absence of cocci is only accepted when the lactic acid bacteria present have significant high nitrate reductase activities. Therefore, if the product fermentation process demands activity of nitrate reductase, the use of the *L. plantarum* strains isolated should be associated with a micrococaceae strain or other lactic acid bacteria strain that exhibit the respective activity.

All isolated cultures showed stability after freeze-drying process after 4 weeks and 6 months of storage at – 20 °C because cells viability status remained at 9 Log cfu.mL^{-1} (Table 2). Further investigations are necessary to show possible changes in cell functionality.

Regarding antagonistic activity, lactic acid bacteria isolated from traditional sausage are probably the best candidates for improving the microbiological safety of these products because they are well adapted to the conditions found in sausages and therefore should be more competitive than lactic acid bacteria from other sources (AMMOR et al., 2005b). The main mechanism

Table 1. Determination of lactic acid (D/L isomers) in fermentation medium (MRS broth) after 16 hours of fermentation and pH around 4.43.

L. plantarum	D – lactic acid			L – lactic acid		
	%	g.L^{-1}	mMol.L^{-1}	%	g.L^{-1}	mMol.L^{-1}
ATCC 8014	1.16	0.010	0.11	98.84	0.841	9.24
AL2	34.70	0.209	2.32	65.28	0.393	4.32
AJ2	33.53	0.058	0.64	66.47	0.115	1.26
AM2	34.66	0.078	0.86	65.33	0.147	1.61
AD3	31.30	0.072	0.79	68.69	0.158	1.74
AN3	29.67	0.067	0.74	70.73	0.159	1.75
AF5	34.32	0.069	0.76	65.68	0.132	1.45
R2	31.13	0.066	0.73	68.86	0.146	1.60

Table 2. Stability of *L. plantarum* strains isolated after freeze-dried process and during storage at – 20 °C.

	Microbial population at – 20 °C (mean values Log cfu.mL^{-1} ± SD)		
Microrganism	initial	4 weeks	6 months
L. plantarum AJ2	8.89 ± 0.2	9.12 ± 0.6	8.95 ± 0.1
L. plantarum AL2	9.86 ± 0.7	9.92 ± 0.2	9.89 ± 0.2
L. plantarum R2	9.23 ± 0.3	8.78 ± 0.3	9.20 ± 0.7
L. plantarum AF5	9.58 ± 0.1	9.65 ± 0.8	9.63 ± 0.6
L. plantarum AD3	9.86 ± 0.2	9.89 ± 0.3	9.81 ± 0.1
L. plantarum AN3	9.76 ± 0.2	9.79 ± 0.2	9.74 ± 0.5
L. plantarum AM2	9.39 ± 0.1	9.25 ± 0.5	8.97 ± 0.3

Table 3. Antagonistic activity of *L. plantarum* strains isolated from artisanal sausage.

	L. plantarum strains isolated						
Reference strains	AL2	AJ2	AD3	R2	AM2	AN3	AF5
S. xylosus ATCC 29971	+	–	+	–	–	+	+
S. xylosus isolated	–	–	+	–	–	+	+
S. aureus ATCC 12598	–	+	–	+	+	–	+
E. coli ATCC 25922	–	–	–	–	–	–	–
L. monocytogenes NTC 098630	+	+	+	+	+	+	+

Symbols for well diffusion assay: (+) large inhibition zone (width ≥ 3.0 mm); (–) no inhibition zone.

by which lactic acid bacteria suppress their competitors is the formation of lactic acid (the most important mechanism of action of protective cultures), acetic acid, and, possibly, bacteriocins (LÜCKE, 2000). Other metabolites of lactic acid bacteria inhibit Gram-negative bacteria *in vitro*, but it is unlikely that they will be exploited to improve the safety and stability of meats. Some are not formed in sufficient amounts (e.g. reuterin), some interfere with the sensory properties (e.g. diacetyl, hydrogen peroxide), and some raise regulatory concern (e.g. benzoic acid) (LÜCKE, 2000).

Bacteriocinogenic lactic acid bacteria have shown effective inhibition of growth of pathogens, such as *L. monocytogenes, S. aureus, Bacillus cereus* and *Clostridium dificile*, even under *in situ* conditions (HOLZAPFEL; GEISEN; SCHILLINGER, 1995). According to Papamanoli et al. (2003), out of seven *L. plantarum* strains isolated from naturally fermented dry sausage, five showed antimicrobial activity against *L. monocytogenes* strains and two inhibited growth of two strains of *S. aureus,* but no inhibition was observed against strains of *E. coli* 0157:H7 and *B. cereus*.

In the present work, seven isolated strains exhibited antagonistic activity against *L. monocytogenes* NTC 098630, four isolated strains inhibited the growth of *S. aureus* ATCC 12598 and *S. xylosus* ATCC 29971, and three isolated strains inhibited the growth of *S. xylosus* strain isolated; no inhibition was observed against *E. coli* ATCC 25922 (Table 3).

The results indicate antagonistic activity of *L. plantarum* isolated strains, but further studies are necessary to define the chemical nature, classification and characterization of these antimicrobial compounds. According to Geisen, Lücke, Kröckel (1992), in order to select a microorganism as starter culture it is also important to consider no inhibition against Gram-positive and catalase-positive cocci (e.g. *S. xylosus*) since this microorganism ensures the sensory quality of fermented sausage.

4 Conclusions

Isolated strains of *L. plantarum* showed effective technological properties as starter culture, but additional studies of those properties in naturally fermented sausages are necessary.

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