academicJournals

Vol. 7(19), pp. 2114-2118, 7 May, 2013
DOI: 10.5897/AJMR2012.2288
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Enzymatic activity, surface hydrophobicity and biogenic amines production in lactic acid bacteria isolated from an artisanal Spanish cheese

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Accepted 19 April, 2013

The enzymatic activities of 24 lactic acid bacteria strains have been studied. Lactobacilli strains exhibited the highest β-galactosidase and aminopeptidase activities. Acid phosphatase activity was high in lactobacilli and lactococci strains. Esterase activity was observed for leuconostoc and lactobacilli strains. The hydrophobicity test and the biogenic amines detection were also tested in some strains selected for their use as autochthonous starters in cheese manufacture. In general, all strains showed degrees of hydrophobicity greater than 70%, and were poor former of histamine and cadaverine and medium putrescine former. Tyramine production was medium in three strains and low in two strains of *Lactococcus*.

Key words: Autochthonous starters, cheese, lactic acid bacteria, biogenic amines, enzymatic activity.

INTRODUCTION

Lactic acid bacteria (LAB) with interesting technological and probiotic properties are used as starters in the manufacture of fermented dairy products, such as milk drinks and yoghourts, and their use in cheese production is a common practice nowadays (Mäkeläinen et al., 2009). The cheese constitutes an adequate habitat for the probiotic bacteria in respect to the other fermented products, contributing to their survival also in the human intestinal tract (Bergamini et al., 2009).

Enzyme substrates are powerful tools used extensively in microbiology to study metabolic pathways of microorganisms and to enumerate and identify microorganisms. The hydrophobicity cellular is a necessary characteristic to aggregation and adhesion of the cells to intestinal epithelium and constitutes a requisite in the probiotic strains (Pérez et al., 1998; Del Re et al., 2000). LAB with decarboxylase activity of amino acids could produce biogenic amines (BA) in fermented foods, as is the cheese. Then, the presence of BA is often inevitable in these products, although it could be conditioned by hygienic and technological aspects (Martuscelli et al., 2005; Curiel et al., 2011). Several authors have reported the presence of putrescine, cadaverine, histamine and tyramine in products in which LAB grow (Arena and Manca de Nadra, 2001; Martuscelli et al., 2005). In the fact, tyramine and histamine are the main biogenic amines in cheese (Burdychova and Komprda, 2007).

The aim of this preliminary study was to evaluate several functional and technological characteristics of LAB strains, isolated from artisanal Genestoso cheese. The enzymatic activities screening by rapid methods

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Abbreviations: BA, Biogenic amines; **MRS,** Man-Rogosa Sharpe; **HPLC,** high performance liquid chromatography.

(API-ZYM system), the ability to produce biogenic amines, the hydrophobicity index and the incompatibility among the strains, were analysed in order to provide information on their approach as adjuncts or starters.

MATERIALS AND METHODS

Lactic acid bacteria strains

Twenty-four strains were chosen on the basis of their antimicrobial activity against four Gram-positive reference strains from 420 strains isolated from Genestoso cheese, an artisanal Spanish raw cow's milk cheese, to study their technological aptitude (González et al., 2007; 2010). In the present work, enzymatic activities using the semi quantitative API-ZYM system were tested for these strains. The hydrophobicity test, the biogenic amines detection and the incompatibility of growth were also tested in five selected strains by their technological aptitude. The strains were cultured in Man-Rogosa Sharpe (MRS) broth at 30°C for 18-24 h before been tested by the following assays.

Enzymatic activity

The enzymatic activity of the LAB strains was evaluated using the API-ZYM system (BioMérieux, Marcy-l'Étoile, France). This activity was measured by comparing the colour developed within five minutes with the API-ZYM colour reaction chart (Durlu-Ozkaya et al., 2001) and the results expressed as nmol of substrate hydrolysed.

Surface hydrophobicity assay

The characteristic of the cell surface was determined following the recommendations of several authors (Pérez et al., 1998; Nostro et al., 2004; Dewan and Tamang, 2007). The cell surface hydrofobicity (H%) was calculated according to Nostro et al. (2004).

Fresh cultures of strains selected as starter culture from Genestoso cheese, were grown in MRS broth at 30°C for 24 h and then centrifuged at 8000 g for 5 min. The pellet was washed three times with 9 mL of Ringer solution and thoroughly mixed. 1 mL of the suspension was taken and the absorbance at 580 nm was measured. Equal volumes of suspension and n-hexadecane were mixed in duplicates and mixed thoroughly. The phases were allowed to separate by decantation for 30 min at room temperature. The aqueous phase was carefully removed and the absorbance at 580 nm was measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrofobicity (H%), which was calculated according to Nostro et al. (2004), which consider "hydrophobic" a percentage hydrophobicity index greater than 70%.

Biogenic amines production

Production of biogenic amines was tested by qualitative and quantitative methods. Qualitative analysis was performed in a different amino acid decarboxylase media described by Bover-Cid and Holzapfel (1999). The media were supplemented with the corresponding precursor amino acids (L-histidine monohydrochloride, L-ornithine monohydrochloride, L-lysine monohydrochloride and tyrosine disodium salt) at 1% final concentration. The production of at least one biogenic amine was recorded by the formation of a purple colour in the decarboxylase broth, according to Curiel et al. (2011). Biogenic amine production was confirmed by analytical quantitative methods, such as high performance liquid chromatography (HPLC). The samples were prepared following the procedures reported by Bover-Cid and Holzapfel (1999) and Burdychova and Komprda (2007). Samples were derivatized by dansyl chloride solution and the analysis were performed according to the methods of Eerola et al. (1993) and Martuscelli et al. (2005).

Detection of incompatibility of the strains

Agar spot test assay was used to study the incompatibility among the lactic acid bacteria, as described by Casla et al. (1996).

RESULTS AND DISCUSSION

None of the 24 strains showed α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase activities (Table 1). LAB strains without β-glucuronidase activity could be considered probiotic flora for human. Lactobacilli and one strain of leuconostoc showed high β-galactosidase activity ($\geq$ 40 nmol substrate hydrolysed). These results agree with those obtained by Mathara et al. (2004) and Dewan and Tamang (2007), who reported that the β-galactosidase is the main enzyme by which homofermentative lactobacilli transform lactose into lactic acid. β-galactosidase contributes to the acidification of dairy products, reduces the intolerance to lactose found in certain human populations and can stimulate the growth and colonization of bifidobacteria with a probiotic effect in the human intestine (Zárate and López-Leiva, 1990). In fact, this enzyme is included in the "Probiotic Active Substance", a concept, introduced by Naidu et al. (1999).

The absence of α-glucosidase activity in lactococci strains concurs with previous studies (Herreros et al., 2003) and may be due to the fact that phospho-β-galactohydrolase is the predominant carbohydratase in *Lactococcus* (Marshall and Law, 1984). This activity was low or not detected in enterococci strains. Alkaline phosphatase activity was very low or not detected for all strains, which agrees with other authors (Herreros et al., 2003). In contrast, acid phosphatase activity was generally high for several strains of lactococci and lactobacilli tested.

Most strains showed an esterase or esterase-lipase activity on short-chain fatty acids, so lipase (C14) activity was not detected or was very low. These results agree with those reported by Ballesteros et al. (2006) and Arenas (2007), who concludes that in this artisanal cheese, the short-chain fatty acids predominate during the first stages of ripening, when LAB reached higher presence in the cheese. The low level of protease activity, the high peptidase activity and the low levels of esterase-lipase (C4 and C8) activity among the LAB strains tested could be desirable traits for flavour and texture development during cheese ripening.

All the 24 strains tested showed Leu-arylamidase activity, reaching high values for some strains.

Table 1. Enzymatic activity[a] detected using API-ZYM system, of whole cells of lactic acid bacteria isolated from Genestoso cheese.

Species	Strain	Enzyme tested[b]																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
*Lactoccocuslactis*subsp. *lactis*	GE 11	-	-	-	-	0-5	-	-	-	-	40	5	-	-	-	-	-	-	-	-
	GE 12	-	-	-	-	20	-	-	0-5	-	0-5	0-5	-	-	-	-	-	-	-	-
	GE 13	-	-	-	-	20	-	-	0-5	-	0-5	0-5	-	-	-	-	-	-	-	-
	GE 18	-	-	-	-	20	-	-	0-5	-	0-5	0-5	-	-	-	-	-	-	-	-
	GE 44	-	-	-	-	0-5	-	-	-	-	40	5	-	-	-	-	-	-	-	-
	GE 61	-	0-5	0-5	-	40	0-5	0-5	-	-	40	-	-	-	-	-	-	-	-	-
	GE 102	-	-	-	-	20	-	-	0-5	-	0-5	0-5	-	-	-	-	-	-	-	-
	GE 103	-	-	-	-	20	0-5	0-5	0-5	-	5	5	-	-	-	-	-	-	-	-
	GE 115	-	-	-	-	40	0-5	0-5	-	-	20	10	-	-	-	-	-	-	-	-
	GE 118	0-5	5	0-5	0-5	40	-	5	-	-	40	0-5	-	-	-	-	-	-	-	-
	GE 2363	-	-	-	-	40	5	5	-	-	40	20	-	-	-	-	-	-	-	-
	GE 2379	-	-	-	-	10	-	-	-	-	30	5	-	-	-	-	-	-	-	-
	GE 2375	-	10	5	-	0-5	-	-	-	-	0-5	30	-	-	-	-	-	-	-	-
Leuconostocmesenteroides	GE 2002	-	20	0-5	-	0-5	0-5	0-5	0-5	-	0-5	5	-	-	-	-	-	-	-	-
Ln. pseudomesenteroides	GE 2003	-	20	0-5	-	0-5	-	-	-	-	0-5	5	-	-	-	-	-	-	-	-
	GE 2068	-	10	-	-	5	-	-	-	-	0-5	0-5	-	≥40	-	-	-	-	-	-
Lactobacillus paracasei	GE 2036	-	10	10	0-5	30	30	5	0-5	5	10	30-40	-	≥40	-	10	0-5	-	-	-
Lb. plantarum	GE 2071	0-5	10-20	5-10	0-5	≥40	≥40	20	-	0-5	≥40	≥40	-	≥40	-	≥40	5	0-5	-	-
	GE 2077	-	-	-	-	≥40	≥40	≥40	-	-	≥40	10	-	≥40	-	-	≥40	30	-	-
Enterococcus faecalis	GE 26	-	20	10	-	20	-	0-5	0-5	0-5	5	5	-	-	-	5	-	-	-	-
	GE 35	-	10	5	-	20	0-5	5	-	0-5	0-5	0-5	-	0-5	-	10	-	-	-	-
	GE 2320	-	10	10	-	5	-	-	-	0-5	10	20	-	-	-	5	-	-	-	-
	GE 2371	-	-	-	-	10	-	-	-	-	30	5	-	-	-	-	-	-	-	-
	GE 2381	-	5-10	5	-	20	-	0-5	-	0-5	5	-	-	0-5	-	5	-	-	-	-

[a]Enzymatic activity expressed as nmol of substrate hydrolysed.[b]Enzymes tested: 1.- Alkaline phosphatase;2, esterase (C4); 3,esterase lipase (C8); 4, lipase (C14); 5, leucinearylamidase; 6, valinearylamidase; 7, cystinearylamidase; 8,trypsin; 9, α-chymotrypsin; 10, acidphosphatase; 11, naphthol-AS-BI-phosphohydrolase; 12,α-galactosidase; 13, β-galactosidase; 14, β-glucuronidase; 15,α-glucosidase; 16, β-glucosidase; 17, N-acetyl-(-glucosaminidase); 18, α-mannosidase; 19,α-fucosidase.

Lactobacillus strains showed the highest aminopeptidase activity and similar behaviour was observed in this genus by other authors (Herreros et al., 2003; Mathara et al., 2004; Ballesteros et al., 2006). All the strains tested showed degrees of hydrophobicity greater than 70%, except *Enterococcus* strain GE 2320 that showed only 69% (Table 2). *Lactobacillus* and *Lactococcus* strains showed the highest hydrophobicity

Table 2. Hydrophobicity (%) and biogenic amines production by the lactic acid bacteria strains isolated from Genestoso cheese[a].

Species	Strain[b]	% Hydrophobicity	Biogenic amines production (µg mL^{-1})[c]			
			Putrescine	Histamine	Cadaverine	Tyramine
Lactococcus lactis subsp. *lactis*	Ge 11	77.93	22.16	7.56	8.40	3.16
	Ge 12	90.09	23.22	n.d.	7.86	2.26
	Ge 2363	85.68	34.54	7.9	6.84	89.26
Lactobacillus paracasei	Ge 2036	87.34	52.42	n.d.	9.02	33.74
Enterococcus faecalis	Ge 2320	69.05	14.48	6.32	8.16	79.20

[a]Values presented are means of two replicates evaluations for each bacterial strain; [b]strains of Genestoso cheese; [c]biogenic amines production in a medium supplemented with the corresponding precursor amino acid; nd, not detected.

results agreed with other authors who studied hydrophobicity of LAB strains isolated from fermented milk products and its correlation with the adhesion ability (Pérez et al., 1998; Dewan and Tamang, 2007). In fact, the adherence is important in the selection of probiotic bacteria (Shah, 2001; Kos et al., 2003). All the strains tested were positive for tyrosine decarboxylase activity using the procedure described by Bover-Cid and Holzapfel (1999) and all the strains, except *Lactobacillus paracasei* Ge 2036, were also positives for histamine. The determination of the amino acid-decarboxylase activity of LAB may result in numerous false negative responses due to the acid production by LAB (Bover-Cid et al., 2001) or to an insufficient growth of the strains. False-positive results could be obtained too in some strains (Curiel et al., 2011). Consequently, the biogenic amines production evaluated by rapid screening methods using the decarboxylase medium was confirmed by HPLC. According to Özogul and Özogul (2007) three categories are defined based in the amine production capacity: “Good amine former” (100-1000 µg mL^{-1}), “medium amine former” (10-100 µg mL^{-1}) and “poor amine former” (< 10 µg mL^{-1}). The results are presented in Table 2.

The production of amines was different among the bacterial species of the same family and even within strains of the same bacterial species, as reported by Özogul and Özogul (2007). The strains tested accumulated little amount of histamine from histidine. However, the strain *Enterococcus faecalis* (Ge 2320) was good histamine and tyramine former but in the enriched broth with other amino acids. Özogul and Özogul (2007) reported that other biogenic amines, more than the amine formed from the amino acid added to the decarboxylase broth, could be produced by the strains, because the medium composition. *Lactobacillus. paracasei* Ge 2036, *Enterococcus faecalis* Ge 2320 and *Lactococcus. lactis* subsp. *lactis* Ge 2363 are medium tyramine former from tyrosine. The accumulation of histamine and tyramine in some specific cheeses or other fermented products has been associated with the presence of LAB (Silla-Santos, 1996; Bover-Cid et al., 2001; Martuscelli et al., 2005). Putrescine and cadaverine have also been detected in large quantities in mature cheeses (Moret et al., 1992). The results of this research work in addition to results obtained in other studies carried out on enzymatic characterization of LAB isolated from Genestoso cheese (González et al., 2010) constitute an essential tool to select LAB strains with interesting characteristics from a technological point of view.

Conclusions

L. lactis subsp. *lactis* Ge 12 and Ge 11 show interesting characteristics from a technological point of view. Consequently, these strains could be tested later in the manufacture of several batches of cheese under controlled conditions in order to design autochthonous starters for the industrial manufacture of artisanal cheeses. There is no doubt that non-starter LAB are essential to the development of the desirable flavours in cheese. Thus, selected strains of non-starter LAB, as *L. paracasei* Ge 2036 strain, selected by their high peptidase activity, could be added as starters or adjuncts in the cheese making process. However, this strain was a medium biogenic amines former for putrescine and tyramine. Its behaviour could be studied in this cheese and, if it would be necessary, other lactobacilli strains could be tested too.

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