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Assessment of lactobacilli strains as yogurt bioprotective cultures

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ABSTRACT

Eleven antifungal *Lactobacillus* strains previously isolated from cow and goat milk were fully characterized using molecular and phenotypic methods. Their antifungal activities were tested in milk and yogurt, against fungal species (*Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Penicillium brevicompactum*, *Rhodotorula mucilaginosa* and *Yarrowia lipolytica*) commonly involved in the spoilage of dairy products. The antifungal strains belonged to *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus zeae* and *Lactobacillus harbinensis* species and showed different acidifying and growth capacities in milk. All tested *Lactobacillus* strains showed an antifungal activity in milk with strain-dependent activity spectra. *Lb. harbinensis* showed a very strong antifungal effect in yogurt by completely inhibiting all tested fungi as compared to control. The other tested strains were much less effective. It is the first time that the antifungal activity of *Lb. harbinensis* is described. This strain is a potential candidate for yogurt biopreservation.

1. Introduction

Dairy products such as yogurts and fermented milks are of high economic importance. A large panel of new products are now proposed to the consumers such as 'juiceceuticals' like fruit-yogurt beverages, or added-value products with low calorie, reduced-fat varieties or supplemented with physiologically active ingredients including fibers, phytosterols, omega-3-fatty acids, whey based ingredients, antioxidant vitamins and isoflavones (Khurana & Kanawjia, 2007). Despite fermented milks and yogurts are generally considered as microbiologically stable, they may be subjected to contaminations with acid tolerant fungi, which can occur at all stages of food processing from raw materials to finished products. *Candida parapsilosis*, *Candida diffluens*, *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Rhodotorula mucilaginosa*, *Yarrowia lipolytica*, *Zygosaccharomyces bailii* or *Penicillium brevicompactum* are among the most frequently encountered fungal contaminants in yogurts and fermented milks, particularly in those containing fruits or sugar (Mayoral et al., 2005). Fungal spoilage is then a major limiting factor for the stability and the commercial value of these products. It causes significant economic losses worldwide and restrains exportation's opportunities. Moreover, evolution of European legislation together with consumers' demand has led industrials to reduce the use of chemical preservatives in fermented dairy products. It is then necessary to find alternative strategies to prevent fungal spoilage and/or to increase their shelf life. In this context, biopreservation, that implies the use of microbial cultures selected for their ability to control the growth of spoilage microorganisms, has taken a considerable development (Mills, Stanton, Hill, & Ross, 2011). Lactic acid bacteria (LAB) that are closely associated with fermented foods, and of which majority possesses the generally recognized as safe (GRAS) status and belongs to the qualified presumption of safety (QPS) list in Europe, are particularly well adapted to dairy products preservation (Bernardeau, Vernoux, Henri-Dubernet, & Guéguen, 2008). LAB are known to possess antimicrobial activities linked to their strong competition for nutrients, to the decrease of pH due to their fermentative metabolism and to the production of inhibitory metabolites. Several species of lactobacilli (*Lb. casei*, *Lb. coryniformis*, *Lb. paracasei*, *Lb. rhamnosus*, *Lb. plantarum*…), pediococci (*P. pentosaceus*, *P. acidilactici*) and lactococci (*Lc. lactis*) have been described as antifungal (Schnürer & Magnusson, 2005). However, few studies (Salih, Sandine, & Ayres, 1990; Schwenninger & Meile, 2004; Suomalainen & Mäyrä-Makinen, 1999; Tawfik, Sharaf, Effat, & Mahanna, 2004) have tested *in situ* the capacity of these bacteria to prevent fungal spoilage in yogurts or fermented milks, in spite of the significant commercial interest in using them as

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natural food preservatives. The objectives of this study were to characterize and to evaluate the potential of 11 *Lactobacillus* strains, previously isolated from cow and goat raw milk, to prevent the growth of common fungal spoilage encountered in fruit yogurts and fermented milks.

2. Materials and methods

2.1. Microorganisms and culture conditions

The 11 antifungal *Lactobacillus* isolates tested in this study were previously obtained from cow and goat milk by Delavenne, Mounier, Déniel, Barbier, and Le Blay (2012). They were assigned to the *Lactobacillus* genus on the basis of their Gram-staining, catalase activity, and partial 16S rRNA gene sequencing. Their antifungal activity was previously confirmed by the overlay method in MRS agar. The reference strains used for identification purposes were *Lactobacillus casei* LMG 6904, *Lb. rhamnosus* ATCC 9595, *Lb. paracasei* LMG 13087, *Lactobacillus zeae* LMG 17315, *Lactobacillus harbinensis* LMG 24040 and *Lactobacillus perolens* LMG 18936. Lactobacilli were conserved in MRS broth supplemented with glycerol 30% (v/v) at −80 °C and reactivated by culturing in 10 ml of MRS overnight at 30 °C.

Eight fungi, commonly encountered in yogurt spoilage (Filtenborg, Frisvad, & Thrane, 1996; Mayoral et al., 2005; Viljoen, 2001), were chosen as indicators for antifungal assays. *D. hansenii* LMSA 2.11.003, *Pichia anomala* LMSA 2.01.001, *R. mucilaginosa* LMSA 2.02.007, *Y. lipolytica* LMSA 2.11.004, *P. brevicompactum* LMSA 1.08.095 and *Penicillium expansum* LMSA 1.08.102 came from the "Souchothèque de Bretagne" culture collection (Université de Brest, Plouzané, France), whereas *Kluyveromyces lactis* CLIB 196 and *K. marxianus* CLIB 282, came from the "Collection de Levures d'Intérêt Biotechnologique (CLIB)" (INRA, Thiverval-Grignon, France). Fungi were stored in yeast extract and malt based medium (YEMA) supplemented with glycerol (30%, v/v) at −80 °C, and cultivated aerobically on YEMA at 25 °C.

2.2. Identification of Lactobacillus spp. isolates

2.2.1. MALDI-TOF mass spectrometry analysis

Bacterial isolates were grown on MRS agar for 48 h at 30 °C and a few colonies were harvested and suspended in 300 µl of sterile distilled water and 900 µl of ethanol (95%). After centrifugation at 13,000*g* for 2 min and elimination of the supernatant, the pellet was diluted in 50 µl of formic acid with 50 µl of acetonitrile (70%) and centrifuged again under the same conditions. The supernatant (1.2 µl) containing the protein extract was spotted in the MALDI sample plate, which was then introduced into the apparatus Microflex LT MALDI-TOF (Bruker Daltonics, Germany). The analysis was performed following the supplier instructions. The produced spectra were collected by the software FlexControl 3.0 and were interpreted by software MALDI Biotyper 2.0 thanks to the database Bruker Taxonomy (Bruker Daltonics, Germany) containing 14 groups and 218 representatives of the *Lactobacillus* genus.

2.2.2. DNA extraction and sequencing of the 16S rRNA, pheS and rpoA genes

Total DNA of each selected isolate was extracted by using the FastDNA® SPIN Kit according to the manufacturer's instructions (MP Biomedicals, Santa Ana, CA). DNA quantity and purity was evaluated using a Nanodrop spectrophotometer (Nanodrop Technology®, Labtech, Palaiseau, France) and used as template for the 16S rRNA, *phe*S and *rpo*A genes amplification. Initial denaturation steps were performed for 5 min at 94 °C for the 16S rRNA gene and for 5 min at 95 °C in the case of *phe*S and *rpo*A genes. Amplifications proceeded for 25 cycles of 60 s at 94 °C, 60 s at 57 °C, 2 min at 72 °C and a final extension step of 5 min at 72 °C for the 16S rRNA gene as described by Zamfir et al. (2006); for 3 cycles at 60 s at 95 °C, 135 s at 46 °C and 75 s at 72 °C followed by 30 cycles of 35 s at 95 °C, 75 s at 46 °C, 75 s at 72 °C and a final extension step of 7 min at 72 °C for the *phe*S and *rpo*A genes as described by Berger et al. (2007) and Naser et al. (2005). Amplicons were sequenced at the Biogenouest sequencing platform in the "Station Biologique de Roscoff" (http://www.sb-roscoff.fr/SG/). Sequences were compared to DNA sequences on the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) and on the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) (November 2011) for species assignment. Phylogenetic analyses were conducted by the neighbor-joining method using MEGA 5.05 software (Tamura et al., 2011). Bacterial sequences for the sequenced strains have been deposited in GenBank and accession numbers are available in Table 1.

2.2.3. Typing of isolates using pulsed-field gel electrophoresis

Isolates of the same species were typed to check for the occurrence of clonal strains. DNA preparation and electrophoresis conditions were performed following the method of Brennan et al. (2002) with some modifications. Plugs were made in the wash solution containing 1 mg lysozyme/ml (Sigma-Aldrich, Saint-Quentin Fallavier, France) and 40 U mutanolysin/ml (Sigma), and 100 U mutanolysin/ml was added to the lysis solution. Slices were washed in 100 µl of restriction buffer (1×) containing bovine serum albumin, for 30 min at 4 °C. They were finally incubated overnight in 100 µl of restriction buffer and 20 U of restriction enzyme/ml at 25 °C with *Sma*I or at 37 °C for *Sfi*I. Electrophoretic conditions for separating the DNA fragments were a pulse time of 0.5–20 s for 12 h and another of 30–60 s for 8 h for DNA digested using *Sfi*I (Rodas, Ferrer, & Pardo, 2005), and a pulse time of 0.5–5 s for 14 h

Table 1
Species assignation of antifungal *Lactobacillus* isolates based on MALDI-TOF analyses and *rpo*A and *phe*S genes sequencing.

Strains	MALDI-TOF	*rpoA* gene sequencing		*pheS* gene sequencing		Species assignation
		Identification	Genbank accession number	Identification	Genbank accession number	
K.V9.3.1Np	*Lb. harbinensis*	*Lb. harbinensis*	JQ774489	nd[b]		*Lb. harbinensis*
M.C7.5.2C	*Lb. casei/zeae*[a]	*Lb. zeae/casei*	JQ774484	*Lb. zeae*	JQ774478	*Lb. zeae*
K.V9.3.1Ng	*Lb. casei/zeae*	*Lb. zeae/casei*	JQ774483	*Lb. zeae*	JQ774477	*Lb. zeae*
M.V9.4.2B	*Lb. paracasei*	*Lb. paracasei*	JQ774487	nd		*Lb. paracasei*
P.C8.7.1A	*Lb. paracasei*	*Lb. paracasei*	JQ774485	nd		*Lb. paracasei*
M.V7.7.1A	*Lb. paracasei*	*Lb. paracasei*	JQ774486	nd		*Lb. paracasei*
K.C8.3.1Hc1	*Lb. paracasei*	*Lb. paracasei*	JQ774488	nd		*Lb. paracasei*
M.V8.6.2F	*Lb. rhamnosus/casei*	*Lb. rhamnosus*	JQ774482	nd		*Lb. rhamnosus*
P.C8.3.2A	*Lb. rhamnosus*	*Lb. rhamnosus*	JQ774481	nd		*Lb. rhamnosus*
M.V7.3.1E	*Lb. rhamnosus/casei*	*Lb. rhamnosus*	JQ774480	nd		*Lb. rhamnosus*
K.C8.3.1I	*Lb. rhamnosus*	*Lb. rhamnosus*	JQ774479	nd		*Lb. rhamnosus*

[a] When two names are proposed, the first is with the highest percentage of identity or the best score.
[b] Not determined.