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Antifungal activity of lactic and propionic acid bacteria and their potential as protective culture in cottage cheese

Benoît Fernandez [a, b], Allison Vimont [a, b], Émilie Desfossés-Foucault [c], Monica Daga [c], Gulshan Arora [c], Ismaïl Fliss [a, b, *]

[a] Institute of Nutrition and Functional Foods, Laval University, 2440, Hochelaga Blvd., Québec, Québec, G1V 0A6, Canada
[b] Food Science Department, Food and Agriculture Faculty, Laval University, 2425 Agriculture St., Québec, Québec, G1V 0A6, Canada
[c] Biena Inc., 2955 Cartier St., St-Hyacinthe, Québec, J2S 1L4, Canada

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ABSTRACT

Dairy products are very susceptible to fungal spoilage, which causes economic losses and is a public health concern due to the possible production of mycotoxins. Several novel approaches have been proposed to delay the growth of mold in cheese. Among these, adding a protective culture is particularly interesting in view of increasing consumer demand for naturally preserved foods. The aim of this study was to select a new protective culture and validate its effectiveness as an inhibitor of fungal proliferation in cottage-type cheese. Food-grade bacteria (88 strains) were screened for inhibition of four spoilage molds commonly isolated from cheese. Strains of *Propionibacterium* and *Lactobacillus* were the most active. Seven strains were selected and tested further, alone and in pairs, for their abilities to prevent *Penicillium chrysogenum* growth in a solidified dairy matrix and in cottage cheese. *Lactobacillus rhamnosus* A238 alone or in combination with *Bifidobacterium animalis* subsp. *lactis* A026 inhibited mold growth for at least 21 days at 6 °C, due probably to the production of secondary metabolites and/or competition for nutrients. Overall, our findings show that these strains inhibit molds, some of them acting in synergy, and have potential for use as bio-preservatives in fresh cheese.

1. Background

Cheese is consumed widely throughout Western Europe and North America, where production reaches respectively 8.6 and 4.8 million tons (Ledenbach & Marshall, 2009). As is the case for many other dairy products, cheese is susceptible to spoilage by microbial contaminants, especially fungi, and in particular species of *Cladosporium*, *Penicillium*, *Mucor*, *Aspergillus* and *Geotrichum* (Ledenbach & Marshall, 2009; Pitt & Hocking, 2009a). The resulting product defects include visible surface growth of mold, which may produce discoloration and highly objectionable musty or bitter off-flavors (Rowe & Donaghy, 2011) and in some cases mycotoxins (Hymery et al., 2014). Therefore, fungal spoilage renders cheese unfit for human consumption and represents huge economic losses for the dairy industry. In Canada, it is estimated that 21% of dairy products are lost to microbial contaminants before they reach a buyer or consumer (Bonti-Akomah, Vignola, & Cahoon, 2015).

Spoilage of cheese by mold can be reduced using pasteurization and chemical preservatives such as sorbates and natamycin (e.g. the Danisco DuPont product Natamax® or the DSM product Delvo®Cid). Physical methods including electron beam irradiation and modified atmosphere packaging have also been tested. However, preventing post-process contamination and retarding the growth of surviving organisms remain a challenge (Ledenbach & Marshall, 2009). Some molds have acquired the ability to degrade sorbate by decarboxylation into *trans*-1,3-pentadiene, causing an off-odor and flavor described as “kerosene-like” (Stopforth, Sofos, & Busta, 2005), or to grow under low oxygen tension (Taniwaki, Hocking, Pitt, & Fleet, 2001).

In view of growing consumer concern with food safety issues, including additive content, the food industry is seeking biological alternatives in order to inhibit undesirable microorganisms and thus extend food shelf life. Among the novel approaches, bio-preservation, and more specifically protective culture, has been

* Corresponding author. Institute of Nutrition and Functional Foods, Laval University, 2440, Hochelaga Blvd., Québec, Québec, G1V 0A6, Canada.
E-mail addresses: benoit.fernandez.1@ulaval.ca (B. Fernandez), allison.vimont.1@ulaval.ca (A. Vimont), emilie@biena.com (É. Desfossés-Foucault), daga@biena.com (M. Daga), arora@biena.com (G. Arora), ismail.fliss@fsaa.ulaval.ca (I. Fliss).

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identified as one of the more promising alternatives. Protective culture refers to food-grade microorganisms able to produce inhibitory molecules and/or to compete with spoilage microorganisms in a food matrix (Alexandraki, Tsakalidou, Papadimitriou, & Holzapfel, 2013). Lactic acid bacteria and propionic acid bacteria are the microbial groups most commonly used as protective cultures, since they are present in fermented foods and have a long history of safe use. Several bio-ingredients are commercially available and being used as inhibitors of spoilage microorganisms to extend dairy product shelf life. HOLDBAC® YM-B Plus and HOLDBAC® YM-C Plus, two commercial protective cultures marketed by Danisco DuPont, both contain co-cultures of *Propionibacterium freudenreichii* subsp. *shermanii* with respectively *Lactobacillus rhamnosus* or *Lactobacillus paracasei* (Fromagex, 2012). The CHR Hansen product FreshQ® contains essentially *Lactobacillus rhamnosus* and *Lactobacillus paracasei* (Danisco DuPont, 2016). A commercial product known as MicroGARD™ (Danisco DuPont) is essentially milk fermented by *P. freudenreichii* subsp. *shermanii* and containing a heat-stable protease-sensitive peptide of 700 Da as well as other metabolites (Al-Zoreky, Ayres, & Sandine, 1991; Salih, Sandine, & Ayres, 1990). Despite the availability of these products, evaluation of the potential of lactic or propionic acid bacteria as anti-fungal protective culture in dairy products remains scant (Cheong et al., 2014; Garcha & Natt, 2012; Lynch et al., 2014; Muhialdin, Hassan, & Sadon, 2011; Schwenninger & Meile, 2004; Zhao, 2011). Inhibition of *Penicillium commune* in cottage cheese by *Lactobacillus plantarum* isolated from various herbs, fruits and vegetables has been reported (Cheong et al., 2014), as has a six-day delay in the growth of *Penicillium expansum* in Cheddar cheese in the presence of *Lactobacillus amylovorus* DSM 19280 (Lynch et al., 2014). The antifungal activity of lactic acid bacteria has been attributed to several compounds, and often to the synergism of organic acids, reuterin, fatty acids, cyclic dipeptides and larger peptides (Crowley, Mahony, & van Sinderen, 2013; Reis, Paula, Casarotti, & Penna, 2012). Acetic, lactic, propionic and phenyllactic (PLA) acids are the most extensively studied organic acids. Other carboxylic acids such as caproic or cinnamic acid and their derivatives also have received attention as antifungal agents (Lynch et al., 2014). Reuterin, a broad-spectrum antimicrobial substance known mainly for antibacterial activity, is produced by several lactobacilli and in increased quantities in the presence of glycerol (Crowley et al., 2013; Nakanishi et al., 2002). Fatty acids and proteinaceous compounds with antifungal properties have also been documented during the past decade. The mechanisms by which these molecules inhibit fungal growth are not yet fully understood (Crowley et al., 2013).

The principal goal of the present study was to screen several bacterial strains for their ability to inhibit molds commonly associated with cheese spoilage. We then sought to evaluate more specifically the potential of the most potent strains to inhibit the growth of *Penicillium chrysogenum* in a solidified dairy matrix and in cottage cheese. Particular attention was paid to the search for synergies between strains.

2. Methods

2.1. Microorganisms and culture conditions

Lactic acid bacteria (80 strains) and propionic acid bacteria (7 strains) and *Bifidobacterium animalis* subsp. *lactis* (1 strain) were obtained from the Biena collection (St-Hyacinthe, QC, Canada). The commercial protective cultures HOLDBAC® LC and HOLDBAC® YM-B (Danisco DuPont) as well as *Lactococcus lactis* subsp. *lactis* ATCC 11454 (American Type Culture Collection, Rockville, MD, USA) and *Pediococcus acidilactici* UL5 (STELA collection, Université Laval, Québec, QC, Canada) were used as controls. The two strains comprising the HOLDBAC® YM-B active portion, namely *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii* JS, were isolated and purified on respectively LAMVAB (Hartemink, Domenech, & Rombouts, 1997) and sodium lactate agar (Huang & Adams, 2004). These strains were stored at −80 °C in MRS (BD Difco laboratories, Sparks, MD, USA) supplemented with glycerol (20%) and cultured routinely in MRS broth at 37 °C under anaerobic conditions for 48 h or 96 h respectively for *Lactobacillus* and *Propionibacterium*. *Bifidobacterium, Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris, Leuconostoc and Pediococcus* were propagated at 30 °C under aerobic conditions for 24 h or for 48 h in the case of *L. lactis* subsp. *cremoris*. *Penicillium chrysogenum* LMA-212, *Mucor racemosus* LMA-722, *Cladosporium herbarum* LMA-929 and *Aspergillus versicolor* LMA-370 were obtained from the STELA collection and used as models of fungal contamination. Mold strains were stored as a spore suspension in 70% sterile glycerol kept at −80 °C and cultured routinely at 22 °C on YEG agar (1% yeast extract, 2% glucose, 1.5% agar) until sporulation. A suspension of spores was prepared in sterile peptone water (0.1% w/v) by sliding the plates back and forth vigorously on the bench top. The concentration of the spore inoculum was adjusted with sterile peptone water to approximately 10^6 spores/mL, based on an optical density of 0.5 at 600 nm (Cheong et al., 2014).

2.2. Screening of bacterial strains for antifungal activity

The antifungal activity assay was based on the overlay method (Rouse, Harnett, Vaughan, & Sinderen, 2008) with some modifications. Briefly, 5 μL of bacterial culture was spotted on MRS agar and incubated under optimal growth conditions. The plates were then overlaid with 8 mL of liquid YEG medium containing 1% agar inoculated with spore suspension to provide approximately 10^4 spores/plate, incubated at 22 °C for 2–5 days until an even layer of mold was visible, and then examined for inhibitory activity. Results were recorded as inhibition zone diameter.

2.3. Determination of the optimal concentration of bacterial strain for antifungal activity and synergy

Strong inhibition observed during the initial screening step was validated in a skim milk matrix using a protocol adapted from an *in vitro* assay of anti-yeast activity of cells suspended in agar (Schwenninger & Meile, 2004) and inspired by the assay of the fractional inhibitory concentration (FIC) index (Punam, 2007) originally used to assess drug interactions. HOLDBAC® YM-B was included as a positive control. The strains were tested alone and in pairs in 24-well plates in order to detect synergy. Bacterial cultures were grown separately as described above, centrifuged (4000×*g* for 5 min), suspended in sterile peptone water (0.1% w/v) at 10^{10} cfu/mL and then diluted serially to 10^6 cfu/mL in sterile peptone water. Each row received 100 μL per well of strain 1 suspended at a concentration of 10^{10} cfu/mL, 10^9 cfu/mL, 10^8 cfu/mL, or 100 μL of sterile peptone water. Each column received similarly 100 μL of strain 2 suspended at the same concentrations or 100 μL of sterile peptone water. Sterile skim milk (12% w/v) in 1% agar at 45 °C (800 μL) was then added to each well and 10 μL of *P. chrysogenum* LMA-212 spore suspension prepared as described above were spotted on the solidified surface, yielding 10^4 spores/spot. Plates were then stored in the dark at 22 °C and observed on days 3, 6, 9, 14 and 21 by two different skilled technicians. Mold was considered inhibited when the well agar surfaces remained shiny and smooth. Bacterial combinations were considered as synergistic when the minimal concentrations leading to mold inhibition were lower than those