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In vitro and *in situ* screening of lactic acid bacteria and propionibacteria antifungal activities against bakery product spoilage molds

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ABSTRACT

In the agri-food context, bioprotective cultures represent an interesting alternative to chemical preservatives. The aim of this study was to evaluate the *in vitro* and *in situ* antifungal activity of lactic acid bacteria (LAB) and propionibacteria against bakery product spoilage fungi. Firstly, the biodiversity of fungal contaminant in pound cakes and milk bread rolls, as well as the resistance to chemical preservatives of representative isolates (n = 21), were studied. *Aspergillus* and *Eurotium* species were the most dominant spoilage fungi and the most resistant towards the tested chemical preservatives. They were followed by *Penicillium*, *Cladosporium* and *Wallemia* spp. Secondly, an *in vitro* screening showed that the most active isolates against selected fungal targets belonged to the *Leuconostoc* spp., *Lactobacillus reuteri* and *Lactobacillus buchneri* groups among the 270 tested LAB, as well as to the *Propionibacterium freudenreichi* and *Propionibacterium acidipropionici* species for the 50 tested propionibacteria. Finally, *in situ* tests showed that cultures of isolates belonging to the *Leuconostoc citreum*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Lactobacillus spicheri*, *L. reuteri* and *Lactobacillus brevis* species could delay one or several target fungal growths after bakery product surface spraying. Moreover, different strain cultures led to delayed fungal growths after incorporation in milk bread rolls preparation. The combination of *in vitro* and *in situ* approaches allowed for the identification of bacteria exhibiting antifungal activity, providing future prospects for use as bioprotective cultures in bakery products.

1. Introduction

Food spoilage is a major concern worldwide and is thought to be responsible for the loss of 5–10% of total food products (Pitt & Hocking, 2009). Food products, being rich in nutrients, can be colonized by specific bacteria and fungi that are adapted to these ecological niches in terms of physiology (temperature, pH, a_w, redox potential) and nutrient assimilation ability. In the context of food spoilage, bakery products correspond to a specific case. Indeed, these food matrices are poorly susceptible to microbial spoilage due to the technology used for their preparation (*i.e.* baking heat-treatment eliminating potential raw material contaminants) and their intrinsic traits (*i.e.* low water activity incompatible with spoilage and pathogenic bacteria growth). However, these products are susceptible to post-treatment aerocontamination by mold spores, including fungi that are well adapted to xerophilic condition (Fustier, Lafond, Champagne, & Lamarche, 1998). Fungal spoilage is usually easy to recognize due to the presence of colonies or thalli at the surface of the product. Fungal development leads to organoleptic degradation not only from an aspect point of view but also, according to the contaminant, to noticeable taste, flavor or texture changes, all preventing product consumption. As a result, fungal contamination leads to shelf life limitation, thus limiting export possibilities and causing consumer dissatisfaction as well as negative brand image. Altogether, fungal spoilage is responsible for substantial economic losses for both industrials and consumers. In the bakery product context, species belonging to the *Penicillium*, *Aspergillus*, *Eurotium*, *Wallemia*, *Fusarium* and *Cladosporium* genera have been identified as the most

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important contaminating molds (Vytřasová, Příbáňová, & Marvanová, 2002).

In order to avoid microbial (including mold) spoilage, and hence extend product shelf life, prevention methods combined with different treatments, also called hurdle technologies, have been applied by the agri-food industries. Prevention methods include the application of good manufacturing and hygiene practices, implementation of hazard analysis critical control point (HACCP) system and the use of air decontamination systems. The food preservation methods can correspond to physical treatments, such as heat treatment, modified atmosphere packaging, cold storage and packaged product irradiation (Farkas, 2001; Pateras, 1998), or to the use of chemical preservatives (*e.g.* organics acid such as acetic, lactic, propionic, sorbic, benzoic acids and their salts). However, food spoilage molds are apparently becoming more and more resistant to chemical preservatives (Schnürer & Magnusson, 2005). In the same time, public authorities are encouraging industrials to limit the use of chemical preservatives (EU, 1995) and to find natural methods for food preservation. This is accompanied by a strong societal demand for "preservative-free" food products as consumers are looking for more natural, less severely processed and safer products (Miranda, Jorge, Dominguez, Cepeda, & Franco, 2011).

In the bakery product context, bioprotective cultures (*i.e.* microorganisms exhibiting antifungal activities) represent a growing interest as an alternative to reduce levels or fully eliminate chemical preservatives. The use of lactic acid bacteria (LAB) and propionibacteria as bioprotective cultures is particularly of interest. Indeed, LAB and propionibacteria are naturally present in many fermented foods and have a long history of safe use as starter cultures in the food industry. Therefore, most of them possess a QPS (Qualified Presumption of Safety) or a GRAS (Generally Recognized As Safe) status. Moreover, certain species or strains are able to produce antifungal compounds including organic acids such as lactic, propionic, acetic, phenyllactic, carboxylic and fatty acids (Lavermicocca et al., 2000), reuterin (Guo et al., 2012), cyclic dipetides (Ström, Sjögren, Broberg, & Schnürer, 2002) or proteinaceous compounds (Coda et al., 2008; Rizzello, Cassone, Coda, & Gobbetti, 2011). In many cases, fungal inhibition results from the additive and/or synergistic activities of several of these compounds (Ryan et al., 2011; Yang & Chang, 2010).

The use of LAB as antifungal bioprotective culture has been reported in many food types (Crowley, Mahony, & Van Sinderen, 2013). For example, Delavenne et al. (2014) showed that the addition of *Lactobacillus harbinensis* K.V9.3.1Np as a bioprotective agent in yogurt was able to totally inhibit yeast contamination without detrimental effect on yogurt organoleptic properties. In sourdough bread, the use of selected LAB has been reported to increase the quality and shelf life of wheat bread (Clarke, Schober, & Arendt, 2002; Corsetti et al. 2000).

In this study, we first investigated the biodiversity of fungal contaminants associated with pound cakes and milk bread rolls as well as the resistance of representative isolates to commonly used chemical preservatives. Then, the antifungal activity of 270 LAB and 50 propionibacteria isolates against 5 fungal contaminants was screened *in vitro*. Finally, the antifungal activities of the most inhibitory strains were evaluated *in situ* i) after surface-spraying of fermented medium at a laboratory scale and ii) after incorporation of fermented medium during pilot preparation of milk bread rolls.

2. Materials and methods

2.1. Isolation and identification of spoilage fungi

The diversity of spoilage molds was studied on 2 bakery products, namely pound cakes and milk bread rolls (French "pains au lait", a type of sweet bread made with sourdough and milk). Naturally spoiled "preservative-free" bakery products were obtained from 2 different bakery product manufacturers. A contaminated piece was removed with a sterile scalpel and deposited at the surface of M2Lev (malt extract, 20 g/l, yeast extract 3 g/l, agar 15 g/l) and M5S5 (malt extract 50 g/l, NaCl 50 g/l, agar 15 g/l) media prior to incubation for 7–10 days at 25 °C and 35 °C. Then, the fungal isolates were purified twice on M2Lev or M5S5 media after incubation for 7 days at 25 °C. A total of 38 isolates were obtained corresponding to 23 and 15 isolates from pound cakes and milk bread rolls, respectively. The isolates were preliminarily characterized using phenotypic methods including macro- and microscopic observations. For genotypic identification, DNA was extracted from mycelial plugs using the 'Fast DNA Spin kit' (MPBio, Illkirch, France) following the manufacturer's instructions. The rDNA internal transcribed spacer (ITS) region (for all isolates), partial β-tubulin (for *Aspergillus* and *Penicillium* spp.) and partial actin genes (for *Cladosporium* spp. from the *sphaerospermum* complex) were PCR-amplified using primers ITS4 and ITS5 (White, Bruns, Lee, & Taylor, 1990), Bt2a and Bt2b (Glass & Donaldson, 1995) and ACT-512F and ACT-783R (Carbone & Kohn, 1999), respectively. Both strand of the obtained amplicons were sequenced at the Biogenouest sequencing platform in the "Station Biologique de Roscoff" (http://www.sb-roscoff.fr/SG/) using the same primers used for PCR amplification. After contig assembly with DNA Baser (Heracle Software, Germany), sequences were compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analyses were performed using sequences from the NCBI database and the MEGA6 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Fungal strains selected as targets for antifungal activity screening were deposited at the "Université de Bretagne Occidentale" culture collection (Plouzané, France).

2.2. Mold resistance towards chemical preservatives

Resistance towards 3 chemical preservatives classically used in bakery products (*e.g.*, potassium sorbate, sodium benzoate and calcium propionate) was evaluated, at pH 5 and 6, for 21 representative isolates of the species encountered in milk bread rolls and pound cakes. Five concentrations of each preservative were tested at pH 5 and 6 with concentrations of 0, 0.0075, 0.015, 0.03 and 0.06 mol/L and 0, 0.03, 0.06, 0.125 and 0.25 mol/L, respectively. After addition of the required preservative amount to M5S5 broth, the pH was adjusted with 1 mol/L NaOH or HCl, and the medium sterilized at 121 °C for 20 min; pH was checked after sterilization. Then, 200 μl of the different media were distributed into 96-well plates followed by inoculation of the tested fungi (20 μl of a 10^5 spores/ml suspension obtained as described previously by Delavenne, Mounier, Déniel, Barbier, and Le Blay (2012). Plates were incubated at 25 °C and fungal growth was visually evaluated after 14 d. Five replicates were performed at each tested concentration and for each strain. The minimum inhibitory concentration (MIC) was determined as the concentration for which no visible growth occurred after the incubation period.

2.3. In vitro screening

2.3.1. Bacteria and media

The antifungal activity of 320 bacterial isolates corresponding to 270 LAB (85 obtained from the LUBEM laboratory (Plouzané, France), 83 from ONIRIS laboratory (Nantes, France), 69 from Bioprox (Levallois-Perret, France) and 33 from milk bread roll sourdough), and 50 propionibacteria from Laboratoires Standa (Caen,