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Identification and quantification of antifungal compounds produced by lactic acid bacteria and propionibacteria

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ABSTRACT

Fungal growth in bakery products represents the most frequent cause of spoilage and leads to economic losses for industrials and consumers. Bacteria, such as lactic acid bacteria and propionibacteria, are commonly known to play an active role in preservation of fermented food, producing a large range of antifungal metabolites. In a previous study (Le Lay et al., 2016), an extensive screening performed both *in vitro* and *in situ* allowed for the selection of bacteria exhibiting an antifungal activity. In the present study, active supernatants against *Penicillium corylophilum* and *Aspergillus niger* were analyzed to identify and quantify the antifungal compounds associated with the observed activity. Supernatant treatments (pH neutralization, heating and addition of proteinase K) suggested that organic acids played the most important role in the antifungal activity of each tested supernatant. Different methods (HPLC, mass spectrometry, colorimetric and enzymatic assays) were then applied to analyze the supernatants and it was shown that the main antifungal compounds corresponded to lactic, acetic and propionic acids, ethanol and hydrogen peroxide, as well as other compounds present at low levels such as phenyllactic, hydroxyphenyllactic, azelaic and caproic acids. Based on these results, various combinations of the identified compounds were used to evaluate their effect on conidial germination and fungal growth of *P. corylophilum* and *Eurotium repens*. Some combinations presented the same activity than the bacterial culture supernatant thus confirming the involvement of the identified molecules in the antifungal activity. The obtained results suggested that acetic acid was mainly responsible for the antifungal activity against *P. corylophilum* and played an important role in *E. repens* inhibition.

1. Introduction

In the bakery product context, moulds are responsible for the spoilage of a variety of products otherwise poorly susceptible to spoilage or pathogen microorganism contaminations due to their preparation (baking treatment) and intrinsic characteristics (low water activity) (Le Lay et al., 2016). These fungal contaminations are responsible for substantial economic losses and may also impact the brand image (Pitt and Hocking, 2009). The most common fungi isolated from bakery products belong to the *Penicillium*, *Aspergillus*, *Eurotium* but also *Mucor*, *Cladosporium* and *Wallemia* genera (Legan, 1993; Le Lay et al., 2016; Poute and Tsen, 1987).

As stated above, fungal contamination is suppressed through baking, but bakery products can be contaminated after this step *via* spores present in the factory air, during cooling, slicing or storage step (Legan, 1993). Therefore, the control of these contaminants is of great importance. Up to now, different methods or combinations of methods (hurdle technologies) have been used to protect food products and extend their shelf-life. These methods include air treatment, modified atmosphere packaging and addition of chemical preservatives (propionate, sorbate, ethanol) (Schnürer and Magnusson, 2005). Regarding the latter compounds, consumers are more and more requesting preservative-free products and therefore natural preservation alternatives, biopreservation being one that was particularly explored as recently reviewed by Axel et al. (2016b).

Lactic acid bacteria (LAB) and propionibacteria are good candidates for biopreservation as they have a long history of safe use. LAB are used in various fermented food like dairy (yogurt, kefir or cheese) and bakery products (sourdough) but also in non-fermented products such as meat, vegetables and seafood (Buckenhüskes et al., 1997; Leistner, 1995; Saithong et al., 2010). Propionibacteria are used as probiotics (Mantere-Alhonen and Makinen, 1987), as well as ripening

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cultures in the production of Swiss type cheese (Biede and Hammond, 1979). During their fermentation, bacteria produce a large spectrum of compounds in food matrices; these metabolites play an important role in the product organoleptic properties but also in preservation (Caplice and Fitzgerald, 1999). Bacterial fermentation generally induces a rapid matrix acidification, due to the production of organic acids, such as lactic, acetic or propionic acids, which play a role in the biopreservation of fermented foods (Batish et al., 1997; Leroy and De Vuyst, 2004). Regarding the antifungal activity of bioprotective cultures, it often results from the synergistic effect of different molecules, organic acids not being the only known active molecules. Indeed, other molecules such as fatty acids (Bergsson et al., 2001, Sjogren et al., 2003), reuterin (Axelsson et al., 1989; Magnusson et al., 2003), cyclic dipeptides (Niku-Paavola, 1999; Strom et al., 2002), and proteinaceous compounds (Coda et al., 2008; Rizzello et al., 2011) may be involved in antifungal activity.

In this study, we characterized the nature of antifungal compounds present in culture supernatants that previously showed an efficient inhibition, both *in vitro* and *in situ*, of bakery fungal contaminants (Le Lay et al., 2016). In parallel, different biochemical methods were used to look for known antifungal metabolites present in active supernatants. Finally, combinations of the identified antifungal metabolites were evaluated to verify if these molecules were indeed responsible for this activity.

2. Materials and methods

2.1. Bacterial strains and culture conditions

In this study, 6 bacteria were selected based on a previous study (Le Lay et al., 2016). Four LAB (*Leuconostoc citreum* L123, *Lactobacillus brevis* Lu35, *Lactobacillus reuteri* 5529 and *Lactobacillus spicheri* O15) and one propionibacteria (*Propionibacterium freudenreichii* LSaci68), all exhibiting antifungal activities *in vitro* and *in situ*, were studied. A negative control, corresponding to a LAB without antifungal activity (*Lactobacillus casei* Lu53), was tested in parallel. Bacteria were grown in wheat flour hydrolysate (WFH) broth as previously described (Le Lay et al., 2016). After incubation at 30 °C for 48 h or 72 h for LAB and propionibacteria, respectively, supernatants were obtained after centrifugation for 10 min at 8422 g and filtration on 0.2 μm pore size PTFE filter (VWR, France).

2.2. Nature of antifungal compounds

Antifungal activity of supernatant was confirmed by pouring in Petri dishes a mix of supernatants obtained after centrifugation and filtration with a 4× concentrated agar solution (75%/25%, v/v). After polymerization, a top layer of PDA medium (Potato Dextrose Broth supplemented with 0.7% agar) was added. Then, 50 conidia of *Penicillium corylophilum* UBOCC-A-112081 or *Aspergillus niger* UBOCC-A-112064 (UBO Culture Collection, Plouzané, France), both isolated from spoiled bakery products (Le Lay et al., 2016), were inoculated in the middle of the Petri dishes and incubated at 25 °C for 7 days. Radial fungal growth was measured every day, with 2 perpendicular measures.

Preliminary characterization of the compounds harboring antifungal activities was performed using a double layer activity test as described above by using supernatants that endured the following treatments: i) neutralization (at pH 7 using 2 M NaOH), ii) heat treatment (90 °C or 120 °C for 20 min) and iii) proteinase K treatment. For the last treatment, supernatants were neutralized at pH 7 followed by digestion with 10 mM of proteinase K for 2 h at 37 °C; at the end of reaction, pH was readjusted to its initial value by using 5 M HCl.

2.3. Quantification of lactic, acetic and propionic acids

Supernatants were centrifuged for 15 min at 7500 g at 4 °C and half diluted with sulfuric acid (0.02 N) then frozen at −20 °C during at least 2 h for protein precipitation. Samples were centrifuged to eliminate the precipitate and were filtered on PVFD 0.45 μm filter. Acetic, lactic and propionic acids were analyzed after injection of 10 μL of the obtained solution in a HPLC Dionex system with an Aminex-A column (Bio-Rad). Organic acid detection was performed at 210 nm using a UV/Visible and a refractometer detectors as described by Thierry et al. (2002).

2.4. Ethanol quantification

Ethanol was quantified with gas chromatography using the internal standard method. Supernatants were first directly injected into the Gas Chromatograph (GC 3900 Varian) equipped with a Chrompack Capillary Column (CP-Sil 8 CB LB/MS CP 8752, 30 m × 0.32 mm, 0.25 μm) for ethanol concentration estimation.

For quantification, 0.02%, 0.05% and 0.2% ethanol standards were prepared and mixed with isopropanol at the same concentration. The same protocol was applied to the tested samples: 500 μL of sample were mixed (v/v) with isopropanol at the same concentrations. Analyses were performed on 1 μL of each mix. Three biological replicates and 3 technical replicates per biological replicate were performed.

2.5. Hydrogen peroxide quantification

These analyses were performed using the OxiSelect Hydrogen Peroxide Assay Kit (Cell Biolabs, Inc., San Diego, USA) following the manufacturer's instructions. A solution at 8.8 mM was first prepared using a stock solution of hydrogen peroxide diluted at 1:1000 in WFH broth. Then, H_2O_2 standard solutions with concentrations ranging from 0 μM to 50 μM were prepared by dilution in WFH medium. Plates were read at 595 nm using a Multiskan FC Microplate Photometer (Thermo Scientific). Sample absorbances were compared to a standard curve and H_2O_2 concentrations were determined.

2.6. Reuterin quantification

Reuterin was measured using the colorimetric method of Circle et al. (1945). Acrolein (Aldrich) standard solutions ranging from 0.05 to 6 mM were prepared in WFH broth medium. To 1 mL of each sample and standard solution, 0.75 mL of a 10 mM tryptophan solution (dissolved in 0.05 M HCl) was added. After homogenization, the mix was incubated at 37 °C for 20 min. The absorbance was recorded at 560 nm using a Genesys 10S UV–Vis spectrophotometer (Thermo scientific). Sample concentrations were determined using linear response of acrolein.

2.7. Antifungal compounds identification by LC-QToF

2.7.1. Standard preparation

Twenty-six compounds (noted A to Z) described in the literature for their antifungal properties (Brosnan et al., 2012) were used (Table 1). Stock solutions at 2 mg/mL were prepared and stored at −20 °C. In parallel, a stock solution corresponding to a mix of the 26 compounds (at 100 μg/mL each) was prepared by diluting the stock solutions with H_2O/acetonitrile (ACN) (90/10, v/v) into amber vials. Standard mixes were prepared at different concentrations, *i.e.*, 1, 5, 10, 30 and 50 ppm, by dilutions of the standard stock solution with H_2O/ACN (90/10, v/v).

2.7.2. Sample preparation

Bacterial supernatants were extracted using the QuEChERS dispersive SPE (dSPE) (Agilent Technologies) as described by Brosnan et al. (2014). Briefly, for each sample, 10 mL of supernatant were mixed in