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Technical note: High-throughput method for antifungal activity screening in a cheese-mimicking model

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ABSTRACT

In this study, we developed a high-throughput antifungal activity screening method using a cheese-mimicking matrix distributed in 24-well plates. This method allowed rapid screening of a large variety of antifungal agent candidates: bacterial fermented ingredients, bacterial isolates, and preservatives. Using the proposed method, we characterized the antifungal activity of 44 lactic acid bacteria (LAB) fermented milk-based ingredients and 23 LAB isolates used as protective cultures against 4 fungal targets (*Mucor racemosus*, *Penicillium commune*, *Galactomyces geotrichum*, and *Yarrowia lipolytica*). We also used this method to determine the minimum inhibitory concentration of a preservative, natamycin, against 9 fungal targets. The results underlined the strain-dependency of LAB antifungal activity, the strong effect of fermentation substrate on this activity, and the effect of the screening medium on natamycin minimum inhibitory concentration. Our method could achieved a screening rate of 1,600 assays per week and can be implemented to evaluate antifungal activity of microorganisms, fermentation products, or purified compounds compatible with dairy technology.
Key words: high-throughput screening, antifungal activity, cheese model

Technical Note

Fungal spoilage of dairy products remains a major concern for dairy manufacturers despite the use of preventive and control approaches, including the use of chemical preservatives. Among methods that are increasingly used by industries for microbial contaminant control, biopreservation is gaining increasing attention due to strong societal (Gerez et al., 2013) and legislative (Fuselli et al., 2012; Stratford et al., 2013) demand for preservative-free products. Biopreservation can be defined as using natural or added microbiota or their antimicrobial compounds to preserve a food product (in terms of safety and quality) and possibly extend its shelf life (Stiles 1996). Lactic acid bacteria (**LAB**) are well known for their ability to exhibit antifungal activity (Schillinger and Villareal, 2010; Wulijideligen and Taku, 2011; Cheong et al., 2014) and protective cultures containing LAB, such as FreshQ (consisting of *Lactobacillus paracasei* and *Lactobacillus rhamnosus* strains; Chr. Hansen, Hørsholm, Denmark) or the HOLDBAC series (consisting of various lactobacilli and propionibacteria; Danisco/DuPont, Madison, WI), are currently used in dairy products for their antifungal properties (Varsha and Nampoothiri, 2016). Recently, Inglin et al. (2015) developed a high-throughput screening method to detect antimicrobial (including antifungal) activities using an agar-spot assay in 24-well plates. This method enabled screening of 2,000 assays per day. However, despite being a useful primary high-throughput screening method for developing protective cultures, this antifungal screening assay used de Man, Rogosa, and Sharpe (**MRS**) agar medium, which can strongly affect expression of antifungal properties and antifungal molecule activity. Indeed, as mentioned in several articles (Stiles et al., 2002; Delavenne et al., 2012; Le Lay et al., 2016), MRS contains acetate, which may reinforce LAB antifungal activity and artificially inflate the number of active isolates. However, assays applied to real food matrices (e.g., dairy product models such as yogurt) can rapidly become labor intensive, thereby reducing the number of isolates that can be screened to detect strains with antifungal activity (Lynch et al., 2014; Delavenne et al., 2015). In addition, these methods do not allow large-scale and rapid screening of antifungal cultures or compounds.

In the present study, we present a high-throughput screening assay for antifungal activity in a cheese-mimicking matrix distributed in a 24-well plate. The proposed method allowed testing the antifungal activity of a large variety of microbial protective compounds:

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bacterial fermentation products, bacterial cultures used as adjunct cultures, and purified antifungal preservatives. To prepare the cheese-mimicking matrix, we used a standardized UF milk retentate (3.4×; 244 g/kg of DM, 65.8 g/kg total fat, 116.2 g/kg of total N, pH 6.53) prepared according to Hannon et al. (2006). Briefly, whole raw cow milk was heated to 50°C and skimmed using a cream separator (Westfalia, Handelsweg, the Netherlands). Skim milk was then microfiltered (0.8-μm Sterilox GP membrane, Pall Corp., Port Washington, NY) at 50°C using the pilot equipment GP7 (Brenet, Mamirolle, France) and cream was heat-treated for 2 min at 95°C followed by fat standardization (20 g/kg final concentration). The retentate was ultrafiltered at 0.02 μm at 50°C (T.I.A., Bollene, France) and sterilized sodium chloride (Sogebul, Sainte-Maure de Touraine, France) was added to reach 0.7% (wt/wt). The salted retentate was heat-treated for 2 min at 95°C (Microthermics, Raleigh, NC), distributed in 1-L sterile bottles, and kept at −20°C until use. Before plate preparation, 10 mL/L of a pH indicator (sterile solution of litmus 50 g/L), 10^6 cfu/L of commercial starter MA016 (*Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis*, Elimeca, Thoissey, France), and 1.5 mL/L of 5× diluted and filtered (0.22 μm) rennet (Danisco, Dangé Saint-Romain, France) were added to the thawed retentate. After vigorous homogenization for 1 min, the retentate was distributed into 24-well plates (2 mL/well) and incubated for 1 h at 30°C and then for 3 d at 20°C, leading to the formation of a "mini-cheese" (pH 5) in each well. The exudate was then removed from the surface of each mini-cheese and plates were stored at 12°C until use.

Using this cheese-mimicking matrix, we screened the antifungal activity of 44 LAB fermented milk-based products and 23 LAB isolates used as protective cultures against 4 fungal targets. The LAB were obtained from the culture collections of CIRM-BIA (Centre International de Ressources Microbienne-Bactéries d'Intérêt Alimentaire, Rennes, France) and LUBEM (Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, Plouzané, France). For fermentation product preparation, a 10%-reconstituted low-heat milk supplemented with 45% anhydrous milk fat and 0.5% litmus (LH medium) was sterilized for 30 min at 110°C. Then, it was individually inoculated with a suspension of the LAB to be tested (1% vol/vol), obtained after 2 subcultures of 24 h at 30°C in MRS broth (Difco, Le Pont de Claix, France). The LH medium inoculated with LAB was then incubated for 20 h at 30°C; 100 μL of the resulting fermentation product was then deposited on the surface of each mini-cheese. After drying for 2 h at room temperature under laminar air flow, plates were surface inoculated with 1 of 4 fungal targets: *Mucor racemosus* UBOCC-A-116002, *Penicillium commune* UBOCC-A-116003, *Galactomyces geotrichum* UBOCC-A-216001, or *Yarrowia lipolytica* UBOCC-A-216006, which were previously isolated from dairy products (Garnier et al., 2017) and obtained from the Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France). Except for yeasts that were first cultured in potato dextrose broth, 10 μL of suspension containing 5×10^3 spores or cells/mL, obtained as previously described (Delavenne et al., 2012), was spotted at the center of each mini-cheese (1 tested fungus/plate). Plates were then incubated at 12°C for 5, 6, and 8 d for *M. racemosus*, *G. geotrichum*, and *P. commune* and *Y. lipolytica*, respectively. Antifungal activity was then determined by visually evaluating fungal growth compared with a negative control without any fermentation product (Figure 1). To test the antifungal activity of LAB isolates for potential use as protective cultures, we applied the same methodology except that LAB isolates to be tested were individually suspended in sterilized milk to reach a final concentration of 10^7 cfu/mL of retentate and inoculated concomitantly with the commercial starter MA016, pH indicator, and rennet before distribution in 24-well plates. In this context, 23 LAB cultures were tested in duplicate against the same fungal targets as those described above, after 2 pre-cultures for 24 h at 30°C in MRS broth. Incubation to obtain the mini-cheeses was performed as described above. Plates were then surface-inoculated with 1 of the 4 fungal targets and antifungal activity was evaluated as described above.

All tested fungal species grew well in the cheese-mimicking model (Supplemental Figure S1; https://doi.org/10.3168/jds.2017-13518) and the results obtained for both replicates were similar, confirming the suitability of this matrix to sustain fungal growth and the reproducibility of the screening method. Among the 46 tested LAB fermentation products, 25 showed antifungal activity against at least one fungal target, and 22 out of the 23 tested protective cultures showed antifungal activity against at least one target. More precisely, intermediate antifungal activities against at least one fungal target were observed for 14 (30%) fermentation products and 12 (55%) isolates, whereas complete inhibition against at least one fungal target was only observed for LAB isolates (n = 10; 45%; Table 1). The most active (++ and +++) isolates or fermentation products corresponded to *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus paracasei*, *Leuconostoc mesenteroides*, and, to a lesser extent, *Lb. rhamnosus*. Antifungal activity varied depending on the utilization mode (protective culture or fermentation product). For example, for *Lb. plantarum* strains tested both for their fermentation products and