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Contribution of volatiles to the antifungal effect of *Lactobacillus paracasei* in defined medium and yogurt

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ABSTRACT

Lactic acid bacteria with antifungal properties can be used to control spoilage of food and feed. Previously, most of the identified metabolites have been isolated from cell-free fermentate of lactic acid bacteria with methods suboptimal for detecting possible contribution from volatiles to the antifungal activity. The role of volatile compounds in the antifungal activity of *Lactobacillus paracasei* DGCC 2132 in a chemically defined interaction medium (CDIM) and yogurt was therefore investigated with a sampling technique minimizing volatile loss. Diacetyl was identified as the major volatile produced by *L. paracasei* DGCC 2132 in CDIM. When the strain was added to a yogurt medium diacetyl as well as other volatiles also increased but the metabolome was more complex. Removal of *L. paracasei* DGCC 2132 cells from CDIM fermentate resulted in loss of both volatiles, including diacetyl, and the antifungal activity towards two strains of *Penicillium* spp. When adding diacetyl to CDIM or yogurt without *L. paracasei* DGCC 2132, marked inhibition was observed. Besides diacetyl, the antifungal properties of acetoin were examined, but no antifungal activity was observed. Overall, the results demonstrate the contribution of diacetyl in the antifungal effect of *L. paracasei* DGCC 2132 and indicate that the importance of volatiles may have been previously underestimated.

1. Introduction

Antifungal lactic acid bacteria (LAB) have been studied in a range of foods and feed like sourdough (Black et al., 2013; Lavermicocca et al., 2000), dairy products (Schwenninger and Meile, 2004) fermented vegetables (Yang and Chang, 2010), and silage (Ström et al., 2002). While most of the efforts have been directed towards finding new potent strains, there is an increasing interest in understanding the antifungal mechanism including the identification and quantification of bioactive compounds produced by these strains. Several recent reviews exist on antifungal compounds produced by LAB (Crowley et al., 2013a; Dalié et al., 2010; Schnürer and Magnusson, 2005; Schwenninger et al., 2011). Phenyllactic acid (PLA) has been reported as an antifungal compound of LAB in several publications. However, Ndagano et al. (2011) found the MIC value of PLA against *Aspergillus* spp. and *Penicillium* sp. to be far higher (180 mM) than the concentrations produced by LAB (0.1–0.5 mM). The same tendency was observed for lactic acid and acetic acid which were produced in concentrations much lower (44.8–76.8 and 1.2–7.5 mM, respectively) than the observed MIC values (>500 mM and 83–125 mM, respectively). This suggests that the antifungal effect is due to synergistic or additive effects of several compounds.

Schwenninger and Meile (2004) described the antifungal properties of a co-culture of *Lactobacillus paracasei* subsp. *paracasei* and *Propionibacterium jensenii* in fermented milk and cheese. They concluded that the inhibition was not solely based on the organic acids produced since acetic and propionic acids did not fully explain the antifungal effect (Schwenninger et al., 2008). Furthermore, some observations indicate a loss of antifungal activity upon cell removal (Schwenninger and Meile, 2004). This could indicate that some of the antifungal compounds disappear in cell-free fermentates, e.g. by being degraded, volatile or being consumed. Bacteria can produce a wide range of volatile organic compounds (Kai et al., 2009; Schulz and Dickschat, 2007). Several studies have shown the antifungal potential of some of these bacterial volatiles. *Pseudomonas* spp. isolated from canola and soybean plants produced the antifungal volatiles cyclohexanol, decanal, 2-Ethyl-1-hexanol, nonanal, benzothiazole and dimethyl

Abbreviations: AL, α-Acetolactate; ALDC, α-Acetolactate decarboxylase; AR, Acetoin reductase; CDIM, Chemically defined interaction medium; C-fermentate, Cell-containing fermentate; CF-fermentate, Cell-free fermentate; GC, Gas chromatography; GUI, Graphical User Interface; LAB, Lactic acid bacteria; LEDs, Light emitting diodes; MEA, Malt extract agar; MIC, Minimal inhibitory concentration; MS, Mass spectrometry; REF, Reference, acidified non-inoculated CDIM; SHS, Static headspace analysis; SPME, Solid phase microextraction; UHT, Ultra-high-temperature.

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trisulfide (Fernando et al., 2005). In another study, unidentified soil bacteria produced the antifungal volatiles trimethylamine, 3-Methyl-2-pentanone, dimethyl disulfide, benzaldehyde and *N*,*N*-Dimethyloctylamine (Chuankun et al., 2004). In spite of this, most of the studies on antifungal compounds produced by LAB strains focus on the non-volatile, liquid cell-free fermentate, often using bioassay-guided fractionation (Ström et al., 2002) and/or pre-concentration, e.g. extraction and drying (Schwenninger et al., 2008). These concentration techniques are well suited for concentrating compounds less volatile than water (or the solvent). However, compounds *more* volatile than water will be lost in the process. The aim of the current study was to examine the role of the major volatile compounds produced by the antifungal *L. paracasei* DGCC 2132. We screened fermentations in both chemically defined interaction medium (CDIM) and milk using qualitative and quantitative methods and minimal sample processing in order to elucidate the volatile profile. Diacetyl was identified as a major volatile compound. The antifungal activity of cell-containing as well as cell-free fermentates was tested towards selected fungal spoilers. The activity of cell-containing fermentate was also investigated after diacetyl formation had been inhibited by converting the precursor enzymatically. The antifungal effect of added diacetyl and acetoin was furthermore examined at concentrations comparable to those produced by *L. paracasei* DGCC 2132. The results help to explain the role of diacetyl in the antifungal effect of *L. paracasei* DGCC 2132 and highlight the likely importance of volatiles.

2. Materials and methods

2.1. Chemicals and materials

2,3-Butanedione (diacetyl) with purity 97%; 3-hydroxy-2-butanone (acetoin) with purity ≥96%; Hydrochloric acid (37%) and 2-hydroxypropionic acid (DL-lactic acid) ≥85% were purchased from Sigma-Aldrich (Schnelldorf, Germany). α-Acetolactate decarboxylase, ALDC (3000 ADU/g) was supplied by DuPont Nutrition Biosciences ApS (Brabrand, Denmark). Tween 80 was from Merck, and UHT Milk was from MILSANI®.

2.2. Microbial strains, media and growth conditions

L. paracasei DGCC 2132 isolated from a dairy matrix was used in these studies. The strain had previously been identified by 16S rRNA sequencing. YO-MIX™ 410 starter culture (DuPont Nutrition Biosciences ApS, Denmark) was used for the production of yogurt. Freeze dried bacteria were stored at −18 °C until use.

Penicillium sp. nov. DCS 1541 (tentative name *Penicillium salamii*, closely related to *Penicillium olsonii*, Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre) and *Penicillium solitum* DCS 302 were used as indicator molds since they had previously been isolated from spoiled fermented dairy products and initial results had shown different sensitivities towards antifungal LAB (unpublished results). Molds were grown on malt extract agar (Galloway and Burgess, 1952) (MEA, 30 g/L malt extract, 5 g/L peptone, 15 g/L agar) for 5–7 days and spores were harvested by adding water containing 0.01% Tween 80 (Merck). The harvested spores were supplemented with 20% glycerol (v/v) and stored at −80 °C until use. All bacteria and mold strains were supplied by DuPont Nutrition Biosciences ApS, Brabrand, Denmark.

2.3. Culture conditions

To test the antifungal activity of *L. paracasei* DGCC 2132 in yogurt, UHT-milk (MILSANI®) was inoculated with 10 DCU/100 L YO-MIX™ 410 starter culture (DuPont Nutrition Biosciences ApS, Denmark) and 10^7 CFU/mL of *L. paracasei* DGCC 2132 followed by fermentation at 43 °C for 7 h. Yogurt without added *L. paracasei* DGCC 2132 was used as control.

A chemically defined interaction medium (CDIM) used for growth of *L. paracasei* DGCC 2132 and antifungal activity tests was prepared based on defined media previously reported for growth of fungi (Andersen et al., 2003; Bockelmann et al., 1999; Emeh and Marth, 1976; Hobot and Jennings, 1981; Meyers and Knight, 1958), LAB (Morishita et al., 1974; Møretrø et al., 1998; Saguir and de Nadra, 2007; Savijoki et al., 2006) and other potential antifungal species such as propionic acid bacteria (Dherbécourt et al., 2008; Glatz and Anderson, 1988).

CDIM (200 mL) was inoculated with *L. paracasei* DGCC 2132 (10^7 CFU/mL) in 250 mL blue cap flasks and fermented at 37 °C for 22 h to obtain a cell-containing fermentate (C-fermentate). pH was measured continuously in batches every 15 min during fermentation (Cinac, Alliance Instruments, Frepillon, France). All batches were made in triplicate. Cell-free fermentates (CF-fermentates) were prepared by centrifugation of C-fermentate (5000 ×g, 15 min at 5 °C) followed by filtration of the supernatant through a 0.45 μm filter (Frisenette, ApS). Un-inoculated CDIM kept at 37 °C for 22 h and acidified with lactic acid to pH 4.5 was used as reference (REF).

REF, C-fermentate, and CF-fermentate were tempered in a 48 °C water bath. After mixing with melted, tempered agar (1%), the media were poured into petri dishes. The plates were used after solidification and a short drying period (<3 h).

2.4. Antifungal activity test

Antifungal activity of *L. paracasei* DGCC 2132 was tested by spotting 20 μL of spore dilution (10^5 spores/mL) of each mold in triplicate on plates of yogurt and plates of REF, C-fermentate and CF-fermentate and incubating at 25 °C for 9 days. Mold growth was documented by recording and analyzing multispectral images with the objective of quantifying area of the mold colonies.

Contribution of volatiles to antifungal activity was assessed in a "plate-on-plate" test system without direct contact between molds and C-fermentate. A REF plate was spotted with 20 μL of a mold spore dilution (10^5 spores/mL). On top of the REF plate a C-fermentate plate or a REF plate (control) was placed upside down and sealed with Parafilm® M. The inhibitory activity was assessed by growth on REF plates after 4 days of incubation at 25 °C.

2.5. Acquisition and analysis of multispectral images

A VideometerLab 2 spectral imaging instrument (Videometer A/S, Hørsholm, Denmark) was used to record objective and reproducible images of the petri dishes with spotted mold. Images were recorded after 2, 3, 4, 5, 6 and 9 days of incubation. To record multispectral images by the VideometerLab, the sample was placed inside the sphere of the instrument (Ulbricht sphere) where diffused light from light emitting diodes (LEDs) was provided at 18 different wavelengths, ranging from 375 to 970 nm. A single-channel image was recorded for each wavelength. The size of all the acquired images was 2056 × 2056 × 18 and the lid of the petri dishes was removed prior to image acquisition to avoid reflection.

The images were subsequently analyzed using PCluster, an in-house MATLAB Graphical User Interface (GUI) developed by Ebrahimi et al. (unpublished results). PCluster is specifically designed for *Penicillium* molds, for which the colonies are often composed of white and green segments; however, its main idea and the concept can be used for quantifying all types of molds. PCluster clusters the pixels in the multispectral images with the objective of quantifying mold growth. The outputs of PCluster are the size (in pixels unit) of the green and white segments of the mold colonies and their average spectra. In the current study, quantification of mold growth was based on the total size (area)