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Short Communication

The use of *Lactobacillus brevis* PS1 to in vitro inhibit the outgrowth of *Fusarium culmorum* and other common *Fusarium* species found on barley

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ABSTRACT

A total of 129 lactic acid bacteria (LAB) were screened for antifungal activity against common *Fusarium* spp. isolated from brewing barley. Four out of the five most inhibiting isolates were identified as *Lactobacillus brevis*, whereas one belonged to *Weissella cibaria. L. brevis* PS1, the isolate showing the largest inhibition spectrum, was selected and the influence of its freeze-dried cell-free supernatant (cfsP) on germination of macroconidia as well as mycelia growth was investigated using *Fusarium culmorum* as target organism. Addition of cfsP into the growth medium at concentrations ≥2% altered the growth morphology of *F. culmorum*, whereas at concentrations >5% the outgrowth of germ tubes from macroconidia was delayed and distorted. The presence of 10% cfsP completely inhibited the outgrowth of *F. culmorum* macroconidia. The activity of the compounds produced by *L. brevis* PS1 was higher at low pH values, i.e. pH<5. Heating and/or proteolytic treatment reduced the inhibitory activity of cfsP, indicating that *L. brevis* produces organic acids and proteinaceous compounds which are active against *Fusarium* spp.

1. Introduction

The mycotoxigenic fungi associated with the human food chain belong mainly to the three genera *Aspergillus*, *Fusarium* and *Penicillium* (Pitt et al., 2000). *Aspergillus* and *Penicillium* species are reported as spoilage organisms from a wide range of food and feeds, whereas *Fusarium* species are often found on cereal grains (Filtenborg et al., 1996; Samson et al., 2000). In grain processing, mycotoxin secretion by storage fungi like *Aspergillus* or *Penicillium* species can be prevented by the selection of appropriate storage conditions of the grains. This approach does not apply for field fungi. Therefore field fungi represent an important threat to the safety of cereal products (Noots et al., 1999). Although processing, notably heat treatment, can reduce mycotoxin concentrations significantly, it does not eliminate them completely (Bullerman and Bianchini, 2007; Ryu et al., 2002). Safety of raw materials and food products can be assured by the use of chemical preservatives. However, during the last decades the application of these compounds has been questioned. In particular, the enhanced interest in natural and free-from foods, preferentially with health-promoting characteristics has forced the food makers to find alternative solutions. The applications of lactic acid bacteria (LAB) as starter cultures as well as their metabolites are a matter of particular interest to perform this task.

LAB have a long history of application in fermented foods because of their beneficial influence on nutritional, organoleptic, and shelf-life characteristics, and are naturally occurring in many food systems (De Vuyst and Leroy, 2007; Tamminen et al., 2004; Vaughan et al., 2001). There is an extensive knowledge about antibacterial compounds, especially bacteriocins, produced by LAB (Aso et al., 2008; De Vuyst and Leroy, 2007; Elegado et al., 2007; Ghrairi et al., 2007; Millette et al., 2008; Oguntoyinbo, 2007) whereas the number of published studies on the identification of antifungal compounds produced by LAB is rather limited. Several low molecular weight compounds, mostly organic acids, have been isolated with the ability to retard or eliminate fungal growth or spores outgrowth, either on their own or synergistically (Batish et al., 1997; Dal Bello et al., 2007; Lavermicocca et al., 2003; Lind et al., 2007; Ryan et al., 2008; Schnurer and Magnusson, 2005). Regarding the great diversity of LAB within a single species, particularly due to environmental adaptions, there is a strong justification for further studies aimed at identifying novel antifungal LAB and characterising the compounds responsible for their inhibitory activity. The ambition of this study was to find LAB isolated from different sources like cheese as well as human, mouse, pig and bovine intestinal sources exhibiting antifungal activity against a variety of important *Fusarium* species commonly found on barley.

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2. Materials and methods

2.1. Fungal cultures and spore–mycelia suspension

Fusarium avenaceum TMW 4.1843, *F. culmorum* TMW 4.2043, *Fusarium graminearum* TMW 4.2046, *Fusarium poae* TMW 4.2044 and *Fusarium tricinctum* TMW 4.1405 all isolates from barley were kindly provided by the culture collection of Lehrstuhl fuer Technische Mikrobiologie, TU-Muenchen Weihenstephan (TMW). Fungi were cultivated on potato-dextrose agar (PDA) plates (Fluka Chemie AG, Buchs, Switzerland) at 25 °C for 5 days and then stored at 4 °C until further use. Small pieces from PDA-plates inoculated with fusaria were transferred into 500 mL of synthetic-nutrient-poor bouillon (SNB) (Nirenberg, 1976). The suspensions were incubated at 25 °C (120 rpm) for 5–7 days to induce both microconidia and macroconidia (*F. avenaceum*, *F. poae* and *F. tricinctum*) or solely macroconidia (*F. culmorum* and *F. graminearum)* formation. Concentrations of 1×10^5 to 3×10^5 CFU per mL were measured by plating out serial dilutions on PDA-plates.

2.2. Bacterial cultures

The LAB used were isolated from cheese as well as human, mouse, pig and bovine intestinal samples. LAB were routinely grown on MRS-agar plates (Fluka Chemie AG, Buchs, Switzerland) under microaerophilic conditions for 48 h at 30 or 37 °C. Long-term storage was done in 35% glycerol at −80 °C.

2.3. Screening for antifungal LAB

The screening of LAB for antifungal activity was performed by nebulising 100 µL of fungal spore–mycelia suspension (approx. 10^4 CFU) onto the surface of petri-dishes containing 20 mL of MRS-agar modified as follows (mMRS): pH adjusted to 6.0, sodium acetate as well as potassium dihydrogenphosphate omitted. After 30 min, bacteria were inoculated as two parallel lines of 3 cm length, keeping a distance between the lines of approx. 2 cm. Plates were incubated under microaerophilic conditions at 30 as well as 37 °C for 48 h followed by an additional incubation for 48 h under aerobic conditions at 25 °C to promote fungal growth. The antifungal activity of each LAB was ascertained by measuring the size of the clear zone surrounding the bacterial streaks. Antifungal activity was scored as follows: −, clear zone size<3 mm; +, clear zone size≥3 mm; ++, clear zone size≥5 mm; and +++, clear zone size≥10 mm. The overall growth of the fungi was compared to that in control plates (i.e. with no LAB present) and rated as follows: a, identical growth of fungi surrounding the clear zone; b, retarded growth of fungi surrounding the clear zone; c, strongly retarded growth of fungi surrounding the clear zone.

2.4. Identification of LAB isolates

Bacteria exhibiting strong antifungal activity were identified upon sequencing of the first 900 bp of the 16 S rDNA (Meroth et al., 2003). To determine the closest relatives of the partial 16 S rDNA sequences, a GeneBank DNA database search was conducted. A similarity of >98% to 16S rDNA sequences of type strains was used as the criterion for identification.

2.5. Production of freeze-dried bacterial supernatant powder

Cell-free supernatant (cfs) powders of the most inhibitory strain (cfsP) and of a non-inhibitory strain belonging to the same species (cfsN) were produced to serve as base material for the experiments describing the nature of the antifungal compounds. Briefly, overnight cultures of bacteria were inoculated in 500 mL of mMRS broth to reach an initial concentration of 10^4 CFU/mL. The bacteria were grown for 144 h at 37 °C (temperature at which the antifungal strain showed its highest activity). Cells were separated from the supernatant by centrifuging twice at 3000 *g* for 15 min at 4 °C. The cell-free supernatant was freeze dried and the powder stored at 4 °C.

2.6. D/L-lactic acid and acetate amount in cfsP and cfsN

The amounts of D/L-lactic acid and acetic acid present in the cfs were determined in solutions containing 25% of cfsP or cfsN redissolved in distilled water. For this purposes D-lactic acid/L-lactic acid as well as acetic acid test kits were used according to the manufacturer instructions (R-biopharm AG, Darmstadt, Germany).

2.7. Impact of bacterial supernatant on growth of F. culmorum

The effect of different concentration of cfs on the growth of *F. culmorum* was examined by using a microplate assay. *F. culmorum* was chosen as the test-fungus due to the fact that it exhibited strong sensitivity during the screening and also it develops macroconidia during spore formation, which simplifies microscopic examinations. Pure macroconidia were obtained by filtering the spore–mycelia suspension of *F. culmorum* through a 30 µm pore size filter paper (Filter Paper 113 wet strengthened, Whatman International Ltd, Maidstone, England). The macroconidia suspension was adjusted to 1.0×10^5 macroconidia per mL. Aliquots of 1 mL were centrifuged at 3000 *g* for 10 min at 4 °C and the supernatant was discarded. The conidia pellets were resuspended in 1 mL malt extract broth (MEB) (Merck KGaA, Darmstadt, Germany). The MEB, adjusted to pH 4 using equal volumes of commercial D/L-lactic acid (Sigma-Aldrich, St. Louis, USA) and variable amounts of 4 M NaOH, contained 0, 1.0, 2.5, 5.0, 7.5 or 10.0% (w/v) of cfsP or cfsN. Before adding to the pellets, the dilutions were filtered through a sterile 0.45 µm MINISART®-plus filter (Sartorius Stedim Biotech GmbH, Goettingen Germany). After resuspending the conidia pellets in MEB, wells of a sterile 96-well microplate (Sarstedt AG & Co, Nuembrecht, Germany) were filled with 200 µL and sealed with optically clear seal for QPCR (Thermo Scientific, Waltham, USA). The microplates were incubated for 48 h at 25 °C and agitated every 4 s for approximately 1 s inside a Multiskan FC microplate-reader (Thermo Scientific, Waltham, USA). The optical density at 620 nm (OD_{620}) was automatically recorded for each well every 3 h. The changes in OD_{620} over time were used to generate *F. culmorum* growth curves at each cfs concentration. The experiment was performed in triplicate.

2.8. Macroconidia germination assay

For both antifungal and control strain, 20 mL of MEB was prepared containing 0 (control), 5 or 10% (w/v) of cfsP or cfsN. The pH was adjusted to 4 as described before. Chemically acidified control (CAC) broths were prepared by adding to MEB the amount of D/L-lactic acid used to adjust the pH and the quantity of D/L-lactic acid and acetic acid present in the cfs of the antifungal strain. The pH was adjusted to 4 using 4 M NaOH. All solutions were filtered through a sterile 0.45 µm MINISART®-plus filter. Macroconidia were obtained as described before but from 20 mL spore and mycelia suspension. After centrifugation at 3000 *g* for 10 min at 4 °C, the macroconidia pellets were resuspended in MEB containing cfs and incubated at 25 °C on an orbital platform shaker Heidolph® Unimax 1010 (Heidolph, Schwabach, Germany) at 350 rpm. Samples of 1 mL were taken at 3 h intervals and centrifuged at 4000 *g* for 3 min. After discarding the supernatant, the pellet was resuspended in 100 µL glycerol:water (50:50 v/v). A volume of 5 µL was transferred onto a microscopy slide and 100 randomly chosen macroconidia were analysed with a HP 630 microscope (Zefa Laborservice GmbH, Muenchen, Germany) at 400× magnification. The number of conidia showing germ tube formation