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Antifungal activity of non-starter lactic acid bacteria isolates from dairy products

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ABSTRACT

The antifungal activity of 81 NSLAB isolates from traditional dairy products against moulds developed on the surface of hard cheeses as well as yeasts was studied. Twenty isolates of facultatively heterofermentative and eleven of obligately heterofermentative lactobacilli from Feta cheese exhibited antifungal activities. These isolates were classified to species level by phenotypic criteria and the SDS–PAGE of whole-cell proteins. The former group also showed a broad spectrum of antibacterial activities with preference towards *Listeria monocytogenes* and other food-borne pathogens. The extracellular antimicrobial substances were sensitive to proteolytic enzymes, suggesting that the inhibitory activity was due to bacteriocin-like substances.

1. Introduction

Lactic acid bacteria (LAB) have traditionally been used as natural biopreservatives in food and animal feed, sauerkraut and silage. Their preserving effect relates mainly to the formation of organic acids and hydrogen peroxide, competition for nutrients and production of antimicrobial substances (Stiles, 1996). Biopreservation refers to extended shelf-life and enhanced safety of foods obtained by the natural or added microflora or their antimicrobial products (Schnürer & Magnusson, 2005). The preserving capacity of bacteria naturally occurring in food has gained increasing interest during the recent years, due to consumers demand for reduced use of chemical preservatives.

Moulds and yeasts are common spoilage organisms of food products, such as cheese. Benzoic acid and sodium benzonate are primarily used as antifungal agents as well as natamycin produced by *Streptomyces natalensis* (Davidson, 2001). However, moulds and yeasts are becoming resistant to antibiotics but also to sorbic and benzoic acids (Brul & Coote, 1999; Viljoen, 2001). Lactic acid bacteria may produce compounds with antifungal activity, such as proteinaceous compounds (Magnusson & Schnürer, 2001), phenyllactic acid and cyclic dipeptides (Ström, Sjögren, Broberg, & Schnürer, 2002) and hydroxylated fatty acids (Sjogren, Magnusson, Broberg, Schnürer, & Kenne, 2003). Bacteriocin-like substances and other low and medium molecular weight mass compounds produced by LAB have been reported as antifungal (Niku-Paavola, Laitila, Matilla-Sandholm, & Haikara, 1999; Okkers, Dicks, Silvester, Joubert, & Odendaal, 1999; Rouse, Harnett, Vaughan, & van Sinderen, 2008). However, studies on the effect of LAB on fungi are complicated by the sensitivity of most fungi to metabolites, lactic and acetic acids (Bonestroo, Dewit, Kusters, & Rombout, 1993).

In the present study, the antifungal effects of NSLAB isolates from Greek traditional cheeses and yoghurt were investigated. For this purpose, the fungal inhibitory spectra of 80 isolates of NSLAB against fungi and yeast strains were screened and an attempt was made to determine the nature of the antimicrobial substances produced by interesting group of isolates.

2. Materials and methods

2.1. Isolates of NSLAB

The isolates of non-starter lactic acid bacteria (NSLAB) used in this study were coming from traditional cheeses and yoghurt and belong to our collection. The eight strains of enterococci and the 10 strains of *Leuconostoc mesenteroides* had been isolated from Teleme cheese from goat milk (Litopoulou-Tzanetaki & Tzanetakis, 1992). Seventeen isolates from various cheeses characterized as *Lactobacillus paracasei* subsp. *paracasei* (Mama, Hatzikamari, Lombardi, Tzanetakis, & Litopoulou-Tzanetaki, 2002) and 15 as *Lactobacillus delbrueckii* subsp. *bulgaricus* (Xanthopoulos, Petridis, & Tzanetakis, 2001) were also used. Twenty isolates obtained from the surface growth of Feta cheese during dry-salting and 11 isolates also from Feta, characterized by simple biochemical criteria (growth at 15 and 45 °C, CO_2 production from glucose and NH_3 from arginine; Sharpe, 1979) as facultatively and obligately heterofermentative lactobacilli, respectively (unpublished data), were also studied. The strains were kept at −80 °C in either MRS broth (rods) or M17 broth (cocci) plus glycerol (70:30).

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2.2. Target strains

The isolates of NSLAB were screened against four mould and two yeast strains. The three mould strains (M1, M4 and MT1) were isolates from the surface of ripening Kasseri cheese, characterized as *Penicillium* spp. by a Key based on the morphology of sporing structures (Harrigan, 1998). *Penicillium candidum* was obtained from Wiesby (Laboratorium "Visby", Tonder aps., DK-6270 Tonder), while the yeasts *Debaryomyces hansenii* strain 29 and *Saccharomyces cerevisiae* strain 51 were isolates from the surface of Feta cheese (Tzanetakis, Hatzikamari, & Litopoulou-Tzanetaki, 1996).

2.3. Fungal inocula

Moulds were grown on malt extract agar slants at 25 °C for several days (until sporulation). The spores were collected after vigorously shaking of slants with sterile peptone water (0.2% w/v). Mycelial debris was removed from spore suspension by filtering twice through several layers of sterile damp cheese cloth (Osman, 2004). Yeast cell inocula were prepared from cultures grown in malt agar slants at 25 °C for 48 h. The growth was collected in sterile peptone water (0.2% w/v) by vigorous shaking. Inocula of both, mould spores and yeasts, (1 ml of the 10^{-1} dilution) were used for the assays.

2.4. Antifungal overlay assay

The antifungal activity of LAB was investigated with an overlay assay (Lind, Jonsson, & Schnürer, 2005; Magnusson & Schnürer, 2001). Bacteria were inoculated in two 2-cm lines on MRS agar plates and allowed to grow at 30 °C. Ten milli liter of soft (7%) malt extract agar containing 1 ml of inoculum of mould and/or yeast was then poured onto the agar plates and incubated at 30 °C. After 48 h, the zone of inhibition was measured. The degree of inhibition was calculated as the area of inhibited growth in relation to the total area of the petri dish and the scale was the following: − = no visible inhibition, (+) = weak inhibition in the soft agar above the bacterial growth, + = no fungal growth on 0.1–3% of plate area/bacterial streak, ++ = no fungal growth on 3–8% of plate area/bacterial streak, +++ = no fungal growth on >8% of plate area/bacterial streak.

2.5. Characterisation of the facultatively and obligately heterofermentative lactobacilli

2.5.1. Characterisation by phenotypic criteria

On the facultatively heterofermentative, Gram-positive, catalase-negative rods the following tests were applied: acid production from cellobiose, glucose, mannitol, melibiose, mannose, raffinose, rhamnose and ribose (Ballows, Trüper, Dworkin, Harder, & Schleifer, 1991; Dellaglio, de Roissart, Torriani, Curk, & Janssens, 1994; Fitzsimons, Cogan, Condon, & Beresford, 1999; Sharpe, 1979). The obligately heterofermentative lactobacilli were classified with the following tests: acid production from arabinose, cellobiose, galactose, mannitol, mannose, melibiose, melezitose, raffinose, rhamnose, ribose, sucrose and xylose (Ballows et al., 1991; Sharpe, 1979). Carbohydrate fermentation was determined using sterile multiwell plates (Greiner Labortechnik, Frickenhousen, Germany) and the methodology suggested by API (BioMerieux SA, 69280 Marcy-l'Etoile, France).

2.5.2. Characterisation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell proteins

The SDS–PAGE of whole-cell proteins was used as an additional taxonomic tool for the characterization of facultatively and obligately heterofermentative lactobacilli. Whole-cell protein analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described elsewhere (Ambadoyiannis, Hatzikamari, Litopoulou-Tzanetaki, & Tzanetakis, 2004). The protein content of the disrupted cells was determined according to Lowry, Rosebrough, Farr, and Randall (1951) and appropriate amounts were subjected to SDS–PAGE according to Laemmli (1970). Registration of the protein electrophoretic patterns, normalization of the densitometric traces, grouping of the strains by the Pearson product-moment correlation coefficient (r) and UPGMA (Unweighted Pair Group Method using Arithmetic Averages) cluster analysis were performed with the software package GelCompar version 4.0 (Applied Maths, Kortzijk, Belgium).

Identification of the isolates was performed by comparison of their protein patterns to the fingerprints of the reference strains. Reference strains of *L. paracasei* subsp. *paracasei* (LMG 11459), *Lactobacillus paraplantarum* (LMG 16673; BCCM/LMG Bacteria Collection, Laboratory of Microbiology, University of Gent, Gent Belgium), *Lactobacillus plantarum* (ATCC 14917; ATCC, American Type Culture Collection, Rockville, Maryland), *Lactobacillus pentosus* (NCFB 363; NCFB, National Collection of Food Bacteria, AFRC, Institute of Food Research, Reading, England), *Lactobacillus rhamnosus* (NCFB 7469), *Lactobacillus brevis* (DSM 2647), *Lactobacillus buchneri* (DSM 5987), *Lactobacillus collinoides* (DSM 20515) and *Lactobacillus fermentum* (DSM 20052; DSMZ, Deutsche Sammlung von Microorganismen and Zellculturen GmbH, Braunschweig, Germany).

2.6. Antimicrobial activity assay-inhibitory spectrum

Activated strains of facultatively and obligately heterofermentative lactobacilli grown for 16 h at 30 °C were tested for inhibitory activity by the method of Kekessy and Piquet (1970), which may detect the production of bacteriocins. Each of the indicator strains was grown in the appropriate broth until an optical density of ~0.45 at 600 nm. The producer strains were inoculated by spot inoculation on the surface of MRS agar plates. After incubation (48 h, 37 °C) the agar was detached from the edges of the Petri dish with a sterile spatula. The plate was then inverted into the lid and the new sterile surface was overlaid by the indicator strains inoculated (1% of the first dilution) into an appropriate soft agar. After incubation the zones of inhibition, whenever formed, were measured. Food-borne pathogens used for testing the antibacterial activities of the strains were: *Escherichia coli* 0:44 NCTC 9702 (NCTC, National Collection of Type Cultures, AFRC, Reading, England), *Staphylococcus aureus* NCTC 9751, *Yersinia enterocolitica* 0:9/4360 (supplied by the Pasteur Institute, Paris), *Listeria monocytogenes* Scott A and a *Bacillus cereus* strain (supplied by the School of Medicine, Aristotle University of Thessaloniki).

2.7. Sensitivity of antimicrobial substances to pH, catalase and proteolytic enzymes

The well diffusion assay (Roy, Batish, Grover, & Neelakantan, 1996) was performed on eight facultatively heterofermentative strains exhibiting both, antifungal and antimicrobial activities with an interesting tendency to inhibit preferentially *L. monocytogenes* Scott A and other food-borne pathogens. Culture extracts from the strains were obtained from MRS broth cultures after centrifugation (12,000*g*, 10 min, 4 °C, Sigma centrifuge, type 3K20). Antimicrobial substances (AMS) samples were neutralized by addition of 5 N NaOH to exclude the organic acid effect. These antibacterial crude preparations were designated as neutralized cell-free-supernatants (NCFS). Catalase (150 IU/ml) was added to neutralized samples to exclude the effect of H_2O_2. The samples were centrifuged again (12,000*g*, 10 min, 40 °C) to remove contaminants.

NCFS were treated with trypsin, pepsin, proteinase K and α-chymotrypsin (Merck, Darmstadt, Germany) to a final concentration of