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Inhibition of *Penicillium nordicum* in MRS medium by lactic acid bacteria isolated from foods

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ABSTRACT

During a screening procedure for lactic acid bacteria exhibiting antifungal activity against an ochratoxin-producing *Penicillium nordicum* (BFE 487), numerous strains were observed to produce zones of inhibition against the mould on MRS agar. For the detection of antifungal activity in the culture supernatants, an assay was used where *P. nordicum* was spotted on agar plates prepared by mixing malt extract agar with the filter-sterilized supernatant. In this "mould agar spot assay" growth of the *Penicillium* was prevented only with supernatants containing the sodium acetate concentration of normal MRS medium and showing a low pH indicating that acetic acid is the main factor involved in inhibition. The comparison of the antifungal effect of culture supernatants from selected LAB strains with un-inoculated MRS medium acidified to the respective pH by addition of HCl or lactic acid showed that the culture supernatants were more effective in inhibiting *P. nordicum* growth than the acidified MRS medium, indicating that besides acetic and lactic acid other metabolic products of the LAB contribute to the inhibition.

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1. Introduction

Strains of *Penicillium nordicum* are commonly isolated from fermented meat products such as cured ham and dairy products such as cheeses and may produce ochratoxin A in these foods (Castella et al., 2002; Geisen, 2004). Modern concepts to reduce the contamination with moulds and production of mycotoxins increasingly involve the application of biopreservation. In particular, lactic acid bacteria are considered to be appropriate organisms to suppress the growth of other microorganisms including yeasts and moulds.

Recent research revealed that LAB can produce a range of antifungal substances including organic acids, proteinaceous compounds and low-molecular weight substances, e.g., phenyllactic acid, reuterin, cyclic peptides, 3-hydroxylated fatty acids, benzoic acid, methylhydantoin, and mevalonolactone (Schnürer & Magnusson, 2005). Examples for the technological application of antifungal LAB in foods are still rare. Recently an antifungal strain of *Lactobacillus plantarum* was used in a sourdough fermentation and the authors of the study reported that the application of a 20% rate of sourdough fermented with this strain showed consistent ability to retard the growth of *Fusarium culmorum* and *Fusarium graminearum* (dal Bello et al., 2007).

During an investigation of the biopreservative and probiotic potential of LAB isolated from various foods, these strains were screened for antifungal activity against *P. nordicum*. The aim of the present study was to further investigate the inhibitory effects observed on agar and to determine the influence of sodium acetate as a major component of the medium on the antifungal activity.

2. Materials and methods

2.1. Cultures and media

A total of 58 LAB strains isolated from various food sources were obtained from different partners (Agricultural University of Athens, Danisco/Denmark, University of Maribor, Slovenia, and Federal Research Centre for Nutrition and Foods, Karlsruhe) involved in an EU project ("Pathogen Combat": Control and prevention of emerging and future pathogens at cellular and molecular level throughout the food chain). Five probiotic LAB strains (*Lactobacillus rhamnosus* LGG, *Lact. casei* Shirota, *Lact. johnsonii* La-1, *Lact. plantarum* 299v and *Lact. plantarum* BFE 5092) and six bioprotective bacteriocin-producing strains (*Lact. sakei* Lb 706, *Enterococcus faecium* BFE 900 and BFE 2207, *Lactococcus lactis* BFE 920, *Carnobacterium maltaromaticum* LV61 and *Carnobacterium divergens* BFE 403) from the culture collection of the BfEL were also included. The mould target strain, *P. nordicum* BFE 487 was also from BfEL. The LAB strains were propagated in MRS (Merck, Darmstadt, Germany) broth at 30 °C or 37 °C (in case of *Lact. johnsonii* La-1). They were

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maintained as frozen stock cultures at −80 °C in MRS broth with 15% glycerol added. *P. nordicum* BFE 487 was cultivated on malt extract agar (ME) (Merck, Darmstadt, Germany) at 25 °C.

Spore inocula were prepared by suspending the conidia in 0.9% NaCl solution with 0.2% Tween 80 and spore concentrations were determined by microscopically counting using a "Neubauer profondeur" cell chamber.

2.2. Screening assay for antifungal activities against *P. nordicum* BFE 487

This assay was a modification of the test system described by Miescher Schwenninger et al. (2005). Briefly, the test strains were spot inoculated onto MRS agar plates and allowed to grow at 30 °C for up to 3 days in anaerobic jars. The plates then were overlaid with malt extract (ME) agar (0.7%, 9 ml) containing about 10^5 spores of *P. nordicum* BFE 487 per ml. After incubation for up to 5 days at 25 °C, the plates were examined for the formation of inhibition zones around the bacterial colonies.

2.3. Preparation of culture supernatants

The LAB strains were grown in MRS broth or modifications of this medium (see Table 1) at 30 °C. After 24 h or longer periods of incubation, cells were centrifuged at 8160g for 10 min. The supernatants were filtered through a 0.2 μm sterile filter (Rotrand, Schleicher & Schüll, Germany) and frozen at −20 °C until use. Tenfold concentrated supernatants of selected strains were prepared by lyophilization.

2.4. Determination of antifungal activity of culture supernatants

In the first assay (spot-on-lawn assay), cell free culture supernatants (10 μl) were spotted onto the surface of MRS agar plates overlaid with 9 ml of ME soft agar (0.7%) which had been inoculated with 1 ml of a spore suspension (10^5 ml^{-1}) of *P. nordicum* BFE 487. After incubation of the plates for three days at 25 °C, they were examined for zones of inhibition.

The second test assay ("mould agar spot assay") was based on the agar spot assay from Cabo, Braber, and Koenraad (2002). Filter-sterilized culture supernatants were mixed with ME agar at 55 °C and poured into plates. Various proportions of supernatants and ME agar were assayed and for the final protocol, small Petri dishes (5.5 cm) were used with a mixture of 6 ml of 1.5-fold concentrated ME agar and 3 ml of the supernatant or the MRS medium adjusted to the same pH. Alternatively, 8 ml of 1.25-fold concentrated ME agar were mixed with 2 ml of the culture supernatant. After drying of the plates, 10 μl of the *P. nordicum* spore suspension (10^6 ml^{-1}) were dropped on the agar, and the plates were incubated for 4–6 days at 25 °C. The diameter of the mould colonies was measured and compared with the control in which the supernatant was replaced by MRS medium. All tests were done in triplicate.

2.5. Growth of LAB strains and production of lactic and acetic acid in modified MRS broth

The following modifications of MRS broth were inoculated with 100 μl of fresh overnight cultures of the test LAB strains: MRS with 61 mmol l^{-1} sodium acetate (= normal MRS), MRS with 12.2 mmol l^{-1} sodium acetate, MRS without acetate and MRS with 0.2% glucose and 61 mmol l^{-1}sodium acetate. The strains were grown at 30 °C for 24 h (in some cases also for 48 h and 72 h). pH was measured using a pH meter (Seven Easy pH S20, Mettler-Toledo, Giessen, Germany) and viable counts were determined by serial dilution and plating on MRS agar. The concentrations of D- and L-lactic acid and of acetic acid of cell-free culture supernatants were determined by spectrophotometric test kits from Roche-Diagnostics (Roche Applied Science, Mannheim, Germany). Cell-free culture supernatants were prepared as described above.

2.6. Growth of *P. nordicum* in the presence of various acetate concentrations and determination of MIC of acetic acid at 4 pH values

MRS broth with sodium acetate at 0, 12.2, 24.4, and 61 mmol l^{-1} was prepared and 10 ml tubes were adjusted to various pH values using 1 N HCl. Following filter-sterilization they were inoculated with 100 μl of a spore suspension of *P. nordicum* BFE 487 (10^5 ml^{-1}). For each pH value, an un-inoculated tube was used as control. The tubes were incubated on a rotary shaker at 25 °C for six days and every day the turbidity was visually recorded.

In the first assay for determination of MIC values, acetic acid was added to malt extract broth giving final concentrations of 0, 2, 5, 10, 20, 30, 40, 50, 80, 100, 200, 300, 400, and 500 mmol l^{-1}. The pH of the medium was adjusted to 3.0, 4.0, 5.0 or 6.0. In the second assay, sodium acetate with final concentrations of 1, 2, 5, 10, and 20 mmol l^{-1} were added to the culture supernatant from *Lact. plantarum* PCS 20 grown for 24 h in MRS broth without acetate. For comparison un-inoculated MRS broth containing the same concentrations of sodium acetate and with the same amount of lactic acid added as determined in the culture supernatant was used. pH of this medium and of the culture supernatant was adjusted to 3.9 using 1 N HCl. Following filter-sterilization, the 10 ml tubes were inoculated with 100 μl of a spore suspension of *P. nordicum* BFE 487 (10^5 ml^{-1}). The tubes were incubated on a rotary shaker at 25 °C and after 5 and 7 days the turbidity was visually recorded. The MIC was defined as the lowest acetic acid concentration where no growth was observed at a given pH.

Table 1
Viable cell numbers of *Lactobacillus plantarum* PCS 20 and *Leuconostoc pseudomesenteroides* PCK 18 in four modifications of MRS broth after 24 h and antifungal activity, pH and concentrations of organic acids in the supernatants.

Strain	Medium	Supernatant				
		Log Cfu ml^{-1}	pH	Lactic acid (mmol l^{-1})	Acetic acid (mmol l^{-1})	Antifungal activity[a]
Lact. plantarum PCS 20	MRS (61 mmol l^{-1} sodium acetate)	9.6	3.92	147.6	68.4	+
	MRS with 12.2 mmol l^{-1} sodium acetate	9.5	3.73	124.3	18.6	–
	MRS without acetate	9.5	3.66	110.6	7.66	–
	MRS with 0.2% glucose	8.7	5.4	25.0	73.8	–
Leuc. pseudomesenteroides PCK 18	MRS (61 mmol l^{-1}sodium acetate)	9.4	4.42	86.7	79.5	+
	MRS with 12.2 mmol l^{-1}sodium acetate	9.3	4.05	85.2	30.8	–
	MRS without acetate	9.3	3.96	76.0	19.6	–
	MRS with 0.2% glucose	9.0	5.95	18.9	74.9	–

All values were means of duplicate determinations.
[a] The antifungal activity of the supernatant was determined using the "mould agar spot assay" with *P. nordicum* BFE 487 as described above.

3. Results

3.1. Screening of LAB strains for antifungal activities

A total of 69 LAB strains including 11 probiotic and protective cultures were screened for inhibitory activity towards *P. nordicum* BFE 487 using an agar spot assay and 37 of the strains were able to produce zones of inhibition on MRS agar plates. These 37 strains included the well known commercial probiotic cultures *Lact. rhamnosus* LGG and *Lact. plantarum* 299v. The size of the inhibition zones increased when the incubation period before overlaying the plates with the mould was extended from 24 to 48 h or 72 h.

In a first attempt to demonstrate antifungal activity in the culture supernatants from the LAB strains, a spot-on-lawn assay was used where 10 μl aliquots were spotted on MRS agar plates overlaid with ME soft agar seeded with *P. nordicum* spores. However, none of the supernatants from the 37 LAB strains screened positive for antifungal activity showed an inhibitory effect towards *P. nordicum*. Similarly, no zones of inhibition were observed with supernatants ten-fold concentrated by lyophilization. As a consequence, a modification of the assay developed by Cabo et al. (2002) was used. In this "mould agar spot assay", filter-sterilized supernatants from 24 h cultures were added to molten ME agar and poured onto plates. After drying the plates these were spot inoculated with *P. nordicum*. In order to establish the conditions allowing demonstration of the inhibitory activity, various ratios of the supernatants and ME agar were assayed. Even with a 1:4 ratio of components (2 ml supernatant + 8 ml ME agar), a retardation or complete inhibition of mould growth was obtained.

3.2. Influence of medium composition on the antifungal activity

It was observed that LAB strains producing large zones of inhibition on MRS agar plates were unable to produce inhibition zones on MRS without sodium acetate and on MRS agar with reduced glucose content. Even after extension of the incubation period to 72 h before overlaying with *P. nordicum*, no zones of inhibition were observed on MRS agar with 0.2% glucose or MRS prepared without sodium acetate.

Therefore the composition of MRS was modified to study the effect of varying amounts of acetate and glucose on the production of organic acids and the antifungal activity of the LAB strains. The homofermentative *Lact. plantarum* PCS 20 producing large zones of inhibition and the heterofermentative *Leuconostoc pseudomesenteroides* PCK 18 showing only a small antifungal effect on MRS agar were selected for these studies. A similar experiment was done with two additional strains (*Lact. plantarum* PCS 22 and *Lact. fermentum* PCK 129). All strains grew well in MRS broth with and without acetate and also in MRS broth with a low glucose content reaching viable counts above 10^9 cfu ml^{-1} after 24 h. However, differences were observed in pH decrease, in the amounts of organic acids produced and in the antifungal activity (see Table 1).

With all strains, an antifungal effect was observed only with normal MRS broth, that means in the presence of 61 mmol l^{-1} sodium acetate. The supernatant from *Lact. plantarum* PCS 20 showed a stronger inhibitory effect as compared to *Leuc. pseudomesenteroides* PCK 18. When the acetate concentration of MRS broth was reduced, no antifungal effect was observed although the amounts of lactic acid produced by the LAB were in a similar range and the pH decrease was even stronger. The lowest pH (3.66) was measured in MRS broth without acetate after growth of strain PCS 20 for 24 h and this supernatant was not inhibitory in the "mould" agar spot assay (Table 1). It became inhibitory when sodium acetate was added to this supernatant to a final concentration of 61 mmol l^{-1}.

These results indicate that the amount of acetate is very essential for the antifungal effect of LAB that can be observed on agar. In addition, a sufficiently low pH is necessary as no antifungal effect was observed with MRS with low glucose content. In MRS with 0.2% glucose, only small amounts of lactic acid were produced and the concomitant pH reduction was low (Table 1).

3.3. Antifungal effect of MRS medium with low pH and with lactic acid added

The "mould agar spot assay" with *P. nordicum* BFE 487 was used to compare the effect of culture supernatants with that of sterile MRS medium acidified to the same low pH and with lactic acid added. When in this assay 2 ml of the supernatant from *Lact. plantarum* PCS 20 (pH 3.92) were mixed with 8 ml ME agar, growth of the mould was completely suppressed on the agar whereas the addition of 2 ml MRS medium of the same pH (3.92) resulted only in a retardation of fungal growth (Fig. 1). When the same amount of lactic acid as determined in the culture supernatant was added to MRS broth (148 mmol l^{-1}) and pH was adjusted to 3.92, the colony diameter was reduced to about 60% as compared to the control (Fig. 1). Similar results were obtained with *Leuc. pseudomesenteroides* PCK 18 and other strains investigated (*Lact. plantarum* PCS 22 and *Lact. fermentum* PCK 129). The amounts of lactic acid produced by *Leuconostoc* PCK 18 and *Lact. fermentum* PCK 129 were smaller and the resulting pH values of the supernatants were higher (around pH 4.4). Nevertheless the supernatants showed a stronger antifungal effect as compared to un-inoculated MRS medium with the respective concentration of D-lactic acid (87 mmol l^{-1} for PCK 18) and the same pH (4.4) (Fig. 2).

3.4. Influence of acetate/acetic acid and low pH on the growth of P. nordicum

The growth of *P. nordicum* BFE 487 was investigated in the presence of various concentrations of sodium acetate. Increasing acetate concentrations resulted in a lower pH tolerance. In MRS broth without acetate added, *P. nordicum* was able to grow even at pH 2.5. When MRS broth contained 12.2 mmol l^{-1} sodium acetate, no mould growth was observed at pH below 3.8. In MRS broth

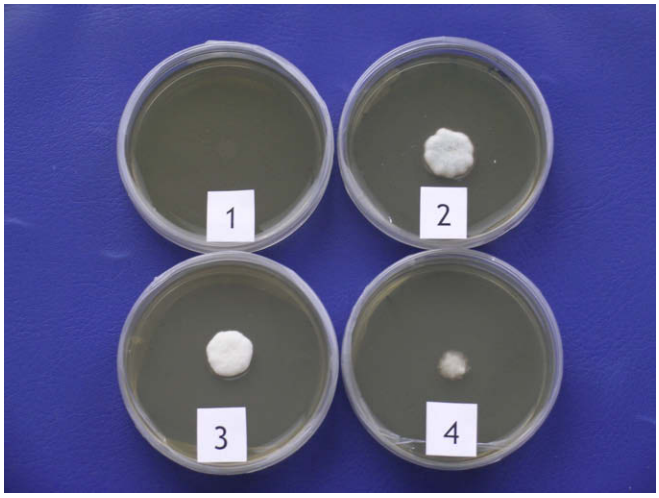

Fig. 1. Growth of *Penicillium nordicum* BFE 487 for 5 days at 25 °C on agar prepared by mixing 8 ml ME agar with 2 ml filter-sterilized culture supernatants from *Lactobacillus plantarum* PCS 20 or 2 ml MRS medium of the respective pH. (1) Culture supernatant from normal MRS (pH 3.92). (2) Culture supernatant from MRS without sodium acetate (pH 3.66). (3) MRS broth adjusted to pH 3.92 by HCl and (4) MRS broth with L-lactate added (144 mmol l^{-1}), pH 3.92.

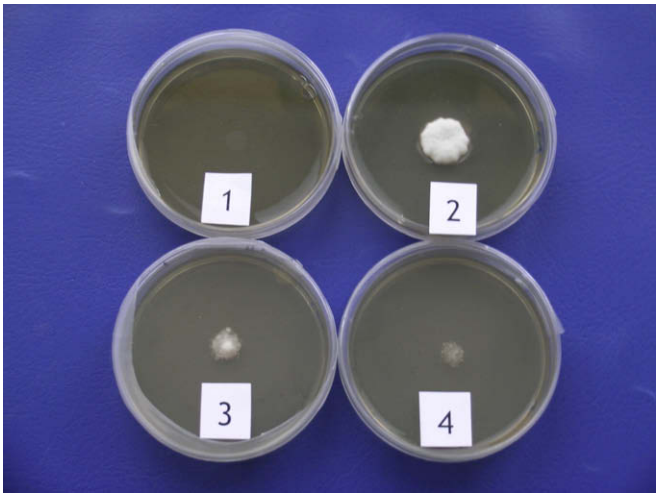

Fig. 2. Growth of *Penicillium nordicum* BFE 487 for 5 days at 25 °C on agar prepared by mixing 6 ml ME agar with 3 ml filter-sterilized culture supernatants from *Leuconostoc pseudomesenteroides* PCK 18 or 3 ml MRS medium of the respective pH. (1) Culture supernatant from normal MRS (pH 4.42). (2) Culture supernatant from MRS without sodium acetate (pH 3.96). (3) MRS broth adjusted to pH 4.42 by HCl and (4) MRS broth with D-lactate added (87 mmol l^{-1}), pH 4.42.

with 30.5 mmol l^{-1} sodium acetate, the minimum pH tolerated for growth was 4.7 and when the acetate concentration was increased to 61 mmol l^{-1}, growth was prevented even at a pH of 5.1. In addition, the lowest concentration of acetic acid allowing growth of *P. nordicum* at 4 pH values was determined with ME broth. The MIC values are given in Table 2. At pH 6.0, high concentrations of acetic acid (>500 mmol l^{-1}) were tolerated whereas at pH 3.0, 10 mmol l^{-1} of acetic acid was sufficient to suppress mould growth.

In addition, MIC values of acetic acid were determined in the presence of the culture supernatant of *Lact. plantarum* PCS 20. For this purpose, increasing amounts of sodium acetate were added to the culture supernatant from PCS 20 grown in MRS broth without sodium acetate for 24 h and with pH 3.9. For comparison, MRS broth of the same pH was prepared and the same amount of lactic acid was added as determined in the culture supernatant (110 mmol l^{-1}). In the presence of this culture supernatant of pH 3.9, 10 mmol l^{-1} of acetic acid were sufficient to inhibit growth of *P. nordicum*. By contrast, a concentration of 20 mmol l^{-1} was required for inhibition in MRS broth adjusted to the same pH and with the same concentration of lactic acid.

4. Discussion

A high percentage of the 58 strains of LAB isolated from various foods and 2 of the 11 probiotic or bioprotective cultures included for comparison were able to produce zones of inhibition against *P. nordicum* BFE 487 on MRS agar. Similar results were obtained by Cabo et al. (2002), Magnusson, Ström, Roos, Sjögren, and Schnürer (2003) and by Miescher Schwenninger et al. (2005) although *P. nordicum* was not included in their investigations. Cabo et al. (2002) suggest from their studies that growth inhibition of their isolates was mainly due to a synergistic effect of the acetic acid present in the medium and the lactic acid produced by the LAB. Our results also indicate that the amount of acetate present in MRS agar indeed is essential for the antifungal effect. All strains producing strong zones of inhibition on normal MRS agar did not show any inhibitory effect on MRS agar plates with reduced acetate content and culture supernatants from these strains grown in MRS broth without sodium acetate did not prevent growth of *P. nordicum* in the "mould agar spot assay". When, on the other hand, sodium acetate was added to such culture supernatants, antifungal activity was observed. The inhibition of moulds by the sodium acetate of MRS medium was also reported by Stiles, Penkar, Plockova, Chumchalova, and Bullerman (2002). *Lact. plantarum* PCS 20 and PCS 22 produced high amounts of lactic acid with a concomitant decrease of pH to 3.9 (which is below the pK_a of 4.75 of acetic acid) during growth in MRS broth. This acidification raises the proportion of the undissociated acetic acid present in MRS medium. According to the classic "weak-acid theory", undissociated organic acid molecules enter the cell and then dissociate within the cell causing progressive decline in the intracellular pH and collapse of the proton motive force (Stratford & Eklund, 2003). This may explain why other strains such as *Leuc. pseudomesenteroides* PCK 18 causing a smaller acidification of MRS medium with a final pH of 4.4 were less inhibitory to the mould.

On the other hand, *Leuc. pseudomesenteroides* PCK 18 is a heterofermentative LAB able to produce acetic acid in addition to D-lactic acid during glucose metabolism. The concentration of acetic acid in the supernatant of this strain was about 12 mmol l^{-1} when grown in MRS broth without acetate. Other heterofermentative LAB such as *Lact. fermentum* PCK 129 produced comparable amounts of acetic acid from glucose in MRS broth. These concentrations, however, were too low to prevent *Penicillium* growth at the final pH of about 4. The determination of the MIC values of acetic acid showed that at least 20 mmol l^{-1} acetic acid are necessary to inhibit *P. nordicum* at pH 4.0. Moreover, it was shown that in MRS broth with 12.2 mmol l^{-1} sodium acetate, a pH reduction below 3.7 would be necessary to inhibit *P. nordicum*. *P. roqueforti* and *P. commune* are reported to be inhibited by 50–80 mmol l^{-1} acetic acid at pH 5.0 (Lind, Jonsson, & Schnürer, 2005).

When the inhibitory effect of the culture supernatants was compared with the anti-mould effect of sterile MRS medium acidified to the same pH and with the same concentration of lactic acid, it was observed that the supernatant was more effective in suppressing growth of *P. nordicum*. Moreover, the minimum concentration of acetic acid needed to inhibit *P. nordicum* at pH 3.9 was reduced in the culture supernatant as compared to MRS medium acidified to the same pH and with the same concentration of lactic acid. These observations indicate that in addition to the synergistic interaction of acetic and lactic acid, other antifungal compounds contribute to the overall inhibitory activity of the LAB strains. These antifungal metabolites may be produced in small concentrations that are not inhibitory in the absence of acetic acid. For instance, phenyllactic acid and 4-hydroxy-phenyllactic acid known to be inhibitory to moulds and yeasts are produced by many LAB including strains of *Lact. plantarum* (Lavermicocca et al., 2000; Ström, Sjögren, Broberg, & Schnürer, 2002; Valerio, Lavermicocca, Pascale, & Visconti, 2004). The concentrations detectable in culture filtrates of the strains, however, are low. Valerio et al. (2004) report on the production of up to 0.57 mmol l^{-1} phenyllactic acid by their strains. On the other hand, at least a concentration of 5 mg ml^{-1} was required for the inhibition of most fungi tested including *Aspergillus* and *Penicillium* (Lavermicocca, Valerio, & Visconti, 2003). Thus phenyllactic acid and 4-hydroxyphenyllactic acid appear to contribute to the overall antifungal effect in synergy with other metabolites (Schnürer & Magnusson, 2005). Antimicrobial

Table 2
MIC-values of acetic acid at pH 3, 4, 5, and 6 for *Penicillium nordicum*.

pH	MIC (mmol l^{-1})
3.0	10
4.0	20
5.0	80
6.0	>500

cyclic dipeptides and 3-hydroxylated fatty acids are other examples for antifungal compounds apparently produced in low amounts by certain LAB and contributing to their antifungal activity (Ström et al., 2002; Sjögren et al., 2003).

It appears that the inhibition of mould growth on an agar plate is the result of a complex interaction of numerous compounds and metabolites contributing to the overall antifungal activity. Many of them are difficult to detect (Schnürer & Magnusson, 2005). The LAB strains involved in this study apparently suppress *P. nordicum* only in the presence of a sufficient amount of acetic acid at a low pH. The contribution of their metabolic compounds to the overall antifungal effect lies in the synergistic interaction with the acetic acid.

In general, for screening of LAB to detect antifungal properties, the application of media containing high amounts of sodium acetate such as MRS broth should be avoided because of the interference of acetic acid with antifungal properties of the strain itself.

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