Control of Mould Growth by *Lactobacillus rhamnosus* VT1 and *Lactobacillus reuteri* CCM 3625 on Milk Agar Plates

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Abstract

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The possibility to control mould growth by *Lactobacillus rhamnosus* VT1 and *Lactobacillus reuteri* CCM 3625 in a milk environment was assessed using the milk agar plate method. Higher antifungal activity was exhibited by actively growing cells of both lactobacilli strains compared with the MRS broth supernatants of both bacterial strains containing metabolites with antifungal activity. The control of mould growth by *Lactobacillus reuteri* CCM 3625 was proved to be associated with the production of the mixture of lactic (0.9% w/w), acetic (0.2% w/w), and succinic (0.2% w/w) acids. The mechanism of mould growth control by *Lactobacillus rhamnosus* VT1 probably consists in the production of lactic acid (1.2% w/w) together with some other metabolite(s) of non-proteinaceous and non-saccharidic nature with antifungal activity.

Keywords: *Lactobacillus*; milk agar plate; mould growth control; antifungal metabolite

The fungal contamination of dairy products such as cheese and fermented milks is a serious problem (VEDAMUTHU 1991; LUND *et al*. 1995). The most common undesirable mould genera originating from air, water, raw materials, packaging material, surfaces of the manufacturing equipment and staff (PITT & HOCKING 1985) are *Penicillium, Fusarium, Mucor, Rhizopus, Aspergillus, Cladosporium, Alternaria* and *Geotrichum* sp. (ROY *et al.* 1996).

Contamination by moulds during dairy production and distribution is directly connected to technological problems, economic loses, and health aspects. It is mainly caused by the decomposition of products, deterioration of organoleptic properties and health risks due to the potential production of mycotoxins or allergenic conidia, ascospores, and mycelia fragments (FILTENBORG 1996).

The fungal growth in the dairy industry was previously controlled mainly by the prevention of mould contamination. This consisted in the use of good manufacturing practices, regulation of inner and outer parameters during the production and storage of dairy products, and in the application of antimycotics (DAESCHEL 1989; DE BOER 1981). Another promising possibility would be the use of combination of these measures with lactic acid bacteria possessing antagonistic activity toward fungi.

A limited number of reports have shown that lactic acid bacteria affect mould growth and mycotoxin production by different mechanisms including production of organic acids (OUWEHAND 1998; BATISH *et al*. 1989) or other heat stable compounds having low molecular weight (NIKU-PAAVOLA *et al*. 1999), depletion of nutrients, or microbial competition (LUND *et al*. 1995). The main objective of this research study was to investigate the interactions between *Lactobacillus rhamnosus* VT1 and *Lactobacillus reuteri* CCM 3625 and several fungal strains in a milk environment simulating the surface of fermented dairy products and to try to characterise antifungal metabolites produced by both lactobacilli strains.

MATERIAL AND METHODS

Microorganisms

- *Lactobacillus rhamnosus* VT1 (i): strain isolated and identified at the Department of Dairy and Fat Technology, Institute of Chemical Technology, Prague.

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• *Lactobacillus reuteri* CCM 3625 (ii): strain obtained from the Czech Collection of Microorganisms, Brno.

The strains are presently kept at the department's culture collection under the catalogue numbers (i) – DMF 30105 and (ii) – DMF 30112. Both strains were used in previous studies (Stiles 1999; Stiles *et al*. 1999). The strains have been subcultured once a week in MRS broth (Oxoid UK) pH 6.2 at 37°C for 18 h, using an inoculum size of 1% v/v.

• *Fusarium* sp. DMF 0101: strain isolated from spoilt processed cheese.
• *Aspergillus* sp. DMF 0801: strain isolated from spoilt fermented dairy product.
• *Penicillium* sp. DMF 0006: strain isolated from spoilt processed cheese.

The fungal strains were isolated at the Department of Dairy and Fat Technology, Prague Institute of Chemical Technology and identified at the Department of Botany, Charles University, Prague. They have been subcultured monthly, using Potato Dextrose Agar – PDA (Oxoid, UK) slants. The incubation was done at room temperature in daylight for 5 to 10 days until a typical appearance, including intensive mycelial growth and sporulation, were achieved.

MRS Broth Supernatants

Both lactobacilli strains were cultivated in MRS broth at 37°C for 18 h. After the cultivation cells were removed by centrifugation (4000 rpm, at 4°C, for 10 min). One part of the supernatant was adjusted to pH 6.0 with 0.1 mol/l NaOH. Both cell-free supernatants, pH adjusted and non-adjusted, were heated in a water bath to 100°C for 5 min. After heat treatment supernatants were cooled and stored at –22°C.

Milk Agar Plate Method

A partially modified method by SUZUKI *et al*. (1991) was used. The modification consisted firstly in a different way of plate inoculation by moulds, secondly in testing the activity of both living lactic acid bacteria and supernatants and organic acids, as is described bellow. Instead of streaking used by Suzuki, a defined amount of mould inoculum was added .

The milk agar plates were prepared as follows: 5 ml of concentrated sterile milk, prepared by dissolving 10 g of skim milk powder (Danone Ltd., CR) in 45 ml distilled water, was inoculated with a loopful of the *Lactobacillus* sp. strain tested. For better growth of lactobacilli 0.5% (w/w) yeast extract (Oxoid, UK) was added to the skim milk. Inoculated milk was solidified with 10 ml of liquid 1.5% w/w bacteriological agar (containing 100 mg/ml of bromcresolepurple – BCP). Ten ml of soft agar (liquid agar containing 0.75% w/w agar and 100 mg/ml BCP) were overlaid. On the surface of soft agar 200 µl of spore suspension of the tested mould strain were inoculated. The spore suspension was prepared by washing freshly cultivated PDA mould slants with 5 ml of sterile peptone/saline diluent containing 0.1% v/v Tween 80. The concentration of spores was adjusted to $A_{600} = 0.3$ in order to obtain approx. 10^{-5} spores/ml.

The method was modified by the addition of supernatant or organic acids into milk with yeast extract instead of inoculation with *Lactobacillus* strain. The cell free supernatant, either with pH adjusted to 6.0, or without any pH adjustment, was added in the amount of 20% v/v.

The following sole acids or acid combinations were added to milk with 0.5% w/w yeast extract: 1% w/w lactic acid (LA) (Sigma-Aldrich, BRD), 0.2% w/w acetic acid (AA), (Penta, CR) and 0.2% w/w succinic acid (SA) (Lachema, CR). In this case *Fusarium* sp. DMF 0101 was used as the indicator strain.

Isotachophoretic Analysis of Organic Acids

The qualitative and quantitative analysis of organic acids produced by each *Lactobacillus* strain in MRS broth and milk with 0.5% w/w yeast extract was done after 18 h of cultivation at 37°C. The sample (1 g) was placed in a volumetric flask, dissolved in distilled water and filled with distilled water up to 100 ml. After filtration the sample was subjected to isotachophoretic analysis using the Isotachophoretic Analyser ZKI-01 (SK) under the following conditions: the leading electrolyte contained 0.01 mol per l HCl and α-aminocaproic acid (Lachema, CR) (pH 4.25), and purified caproic acid (Lachema, CR) of pH 4.5 was used as the terminating electrolyte. An electric current in the pre-separative column of 25 µA and in the analytical column of 50 µA was applied. The evaluation was done using the calibration curve and the concentration of each acid was expressed in % w/w. Analyses were repeated at least twice and the results presented in Table 2 are the means of all replications.

Effect of Enzymes on the Antifungal Metabolites

The character of antifungal metabolites of both *Lactobacillus* strains was determined by their reaction with 4 different enzymes: 3 proteolytic and 1 saccharolytic. Solutions of trypsin, pepsin, pronase E and α-amylase (Fluka, Switzerland) were prepared in phosphate buffer at optimal pH values for each enzyme. The sets of cell-free *Lactobacillus* MRS supernatants were treated with enzymes (having a concentration of 1 mg/g) and incubated at 37°C for 2 h. MRS cell-free supernatants of each *Lactobacillus* strain adjusted to pH 6.0 and heat treated were used as a control. After the incubation pH value of each supernatant was adjusted back to 6.0. The remaining antifungal activity was detected by the well agar diffusion method. The agar plates were prepared using 15 ml PDA, 200 µl of the spore suspension of *Fusarium* sp. DMF 0101 was inoculated onto the surface of each plate. After the inoculation a well (12 mm in diameter) was cut into the

centre of each plate and 200 µl of the tested supernatant were pipetted into the well. After 3 days of cultivation at room temperature the size of the zone produced was measured. The effect of enzymes on supernatants was tested twice, the results given in Table 4 are the means of these two tests.

RESULTS AND DISCUSSION

Antifungal properties of two *Lactobacillus* strains and the nature of their metabolites were further characterized in this study. The partially modified method by SUZUKI *et al*. (1991) was used to observe the interactions between two *Lactobacillus* strains and three mould strains in a milk environment simulating the surface of fermented milk products. The results expressed in Table 1 document that the actively growing cells of both *Lactobacillus rhamnosus* VT1 and *Lactobacillus reuteri* CCM 3625 strains showed more pronounced antifungal activity than 20% v/v MRS broth cell-free supernatants added to milk. As is obvious from Table 1, the growing cells of *Lactobacillus reuteri* CCM 3625 as well as the addition of 20% v/v MRS broth supernatant of this strain caused a total growth inhibition of *Fusarium* sp. DMF 0101, while only actively growing cells of *Lactobacillus rhamnosus* VT1 showed the same effect. There was nearly no difference in the activity of respective supernatants differing in pH (adjusted to pH 6 or approx. 4–4.5 after incubation). Similarly, various authors found that members of facultatively heterofermentative lactobacilli exhibited higher antifungal activity than homofermentative lactobacilli: VANDENBERG *et al*. (1988) in *Lactobacillus rhamnosus* NRRL-B-15972, Suzuki *et al.* (1991) in *Lactobacillus casei* subsp. *casei* ATCC 393, *Lactobacillus rhamnosus* ATCC 7469, *Lactobacillus plantarum* 102, and *Lactobacillus plantarum* 135, and NIKU-PAAVOLA *et al.* (1999) in *Lactobacillus plantarum* VTTE-78076. Strong antifungal activity of obligately heterofermentative *Lactobacillus reuteri* strains was already described (AXELSSON *et al*. 1989; LINDGREN & DOBROGOSZ 1990).

Out of the three mould strains tested, *Fusarium* sp. DMF 0101 was the most sensitive; its growth was totally suppressed by actively growing cells of both lactobacilli strains. The growth of *Penicillium* sp. DMF 0006 and *Aspergillus* sp. DMF 0801 was only partially suppressed, but neither of these moulds ever produced any vegetative spores. *Fusarium* sp. DMF 0101 was therefore chosen for further experiments as a test strain.

Based on previous results documenting the antifungal effectiveness of organic acids produced by lactic acid bacteria (OUWEHAND 1998; BATISH *et al*. 1989), both *Lactobacillus* strains were tested by isotachophoretic assay for the production of organic acids. The results are presented in Table 2 and document well that facultatively heterofermentative strain *Lactobacillus rhamnosus* VT1 produced only lactic acid (utilising glucose in MRS broth and lactose in milk) whereas the obligately heterofermentative *Lactobacillus reuteri* CCM 3625 produced the mixture of lactic, acetic and succinic acids both in MRS broth and in milk.

In order to see whether the organic acids possess any antifungal activity, the effect of sole organic acids or their combinations was tested using the milk agar plate method and *Fusarium* sp. DMF 0101 as a test strain. Table 3 shows that maximum antifungal activity was exhibited by acetic acid alone or in combination with lactic acid and/

Table 1. Antifungal activity of *Lactobacillus rhamnosus* VT1 and *Lactobacillus reuteri* CCM 3625 (growing cells and MRS broth cell-free supernatants) tested by the Milk Agar Plate Method against three fungal strains (observation after 3 day cultivation at room temperature)

Lactobacillus sp. strain	Mould strain	Growing cells milk with 0.5% w/w YE	Milk + 20% v/v MRS broth	
			supernatant	supernatant pH 6.0
Lactobacillus rhamnosus VT1	*Fusarium* sp.	–	+	+
	Penicillium sp.	++	++	++
	Aspergillus sp.	++	+++	+++
Lactobacillus reuteri CCM 3625	*Fusarium* sp.	–	–	–
	Penicillium sp.	++	++	++
	Aspergillus sp.	++	+++	+++
Control	*Fusarium* sp.	+++	+++	+++
	Penicillium sp.	+++	+++	+++
	Aspergillus sp.	+++	+++	+++

– very strong mould growth inhibition (no mycelium present)
+ strong mould growth inhibition (some mycelium present)
++ weak mould growth inhibition (obvious mycelium growth covering more than 25% of plate surface), no spore formation
+++ no inhibition (visible growth of mycelium, vegetative spores present)

Table 2. Amount of organic acids (in % w/w) produced by *Lactobacillus rhamnosus* VT1 and *Lactobacillus reuteri* CCM 3625 in MRS broth and milk with 0.5% w/w yeast extract

Lactobacillus sp. strain	MRS broth			Milk + 0.5% w/w yeast extract		
	Lactic acid	Acetic acid	Succinic acid	Lactic acid	Acetic acid	Succinic acid
Lactobacillus rhamnosus VT1	1.18	0	0	0.88	0	0
Lactobacillus reuteri CCM 3625	0.88	0.20	0.18	0.82	0.22	0.11

Table 3. Inhibition of *Fusarium* sp. DMF 0101 on milk agar plates with 0.5% w/w yeast extract and added organic acids in concentrations: 1% w/w lactic acid (LA), 0.2% w/w acetic acid (AA) and 0.2% w/w succinic acid (SA) alone or in combinations

Days of observation	*LA*	*AA*	*SA*	*LA + AA*	*LA + SA*	AA + SA	LA + AA + SA	Control
2	–	–	+	–	–	–	–	+
4	++	–	+++	–	++	–	–	++
8	+++	–	+++	–	+++	–	–	+++

– very strong mould growth inhibition (no mycelium present)
+ strong mould growth inhibition (some mycelium present)
++ weak mould growth inhibition (obvious mycelium growth covering more than 25% of plate surface), no spore formation
+++ no inhibition (visible growth of mycelium, vegetative spores present)

Table 4. Effect of enzymes on antifungal activity of MRS broth supernatants of *Lactobacillus rhamnosus* VT1 and *Lactobacillus reuteri* CCM 3625 tested by well agar diffusion method on *Fusarium* sp. DMF 0101 (inhibition zones read after 3 day cultivation at room temperature)

Lactobacillus sp. strain	Inhibition zone (mm)				
	Trypsine	Pepsine	Pronase E	α-amylase	Control
Lactobacillus rhamnosus VT1	25	25	29	28	27
Lactobacillus reuteri CCM 3625	18	18	19	20	20

or succinic acid in concentrations that are produced by *Lactobacillus reuteri* CCM 3625 strain. The activity of lactic acid alone in quantity produced by *Lactobacillus rhamnosus* VT1 was not sufficient to suppress the growth of test mould like actively growing cells of this strain. Thus it is probable that *Lactobacillus reuteri* CCM 3625, a species with well described antimicrobial (antibacterial and antifungal) activity, was able to suppress the growth of the mould test strain by the mere production of a mixture of organic acids. On the other hand, the *Lactobacillus rhamnosus* VT1 strain probably produced some other antifungal metabolite(s) that acted concurrently with lactic acid and totally suppressed the growth of *Fusarium* sp. DMF 0101.

We tried further to characterise the nature of the metabolites with antifungal activity of both lactobacilli strains by treating MRS broth supernatants with proteolytic and saccharolytic enzymes. As is documented in Table 4, the antifungal activity of neither of the strains was influenced by the use of these enzymes. This indicates that they are neither of proteinaceous nor of sacharidic character. The characterisation of antifungal metabolites of both strains is still in progress.

Antifungal activity seems to be a promising advantage of both lactobacilli strains, considering their potential applications in dairy technologies. *Lactobacillus rhamnosus* VT1, after testing other important metabolic activities (acidifying, proteolytic), may be used for cheese production as a part of the so called NSLAB (non starter lactic acid bacteria), *Lactobacillus reuteri* 3265, to produce fermented milks.

References

AXELSSON L., CHUNG T.C., DOBROGOSZ W.J., LINDGREN E.E. (1989): Production of a broad spectrum antimicrobial substance by *Lactobacillus reuteri.* Microbial Ecol. Health Dis., **2**: 131–136.

BATISH V.K., GROVER S., LAL R. (1989): Screening lactic starter cultures for antifungal activity. Cult. Dairy Prod. J., **24**: 21–25.

DE BOER E. (1981): Food preservatives. In: SAMSON R.A., HOEKSTRA E.S., VAN OORSCHOT C.A.N. (Eds.): Introduction to Food–borne Fungi. Baarn, The Netherlands, Centralbureau voor Schimmelcultures: 232–235.

DAESCHEL M.A. (1989): Antimicrobial substances from lactic acid bacteria used as food preservatives. Food Technol., **43**: 164–167.

FILTENBORG O., FRISVALD J.C., TRANE U. (1996): Moulds in food spoilage. Int. J. Food Microbiol., **33**: 85–102.

LINDGREN S.E., DOBROGOSZ W.J. (1990): Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev., **87**: 149–164.

LUND F., FILTENBORG O., FRISVALD J.C. (1995): Associated mycoflora of cheese. Food Microbiol., **12**: 173–180.

NIKU-PAAVOLA M.L., LAITILA A., MATTILA-SANDHOLM T., HAIKARA A. (1999): New types of antimicrobial compounds produced by *Lactobacillus plantarum.* J. Appl. Microbiol., **86**: 29–35.

OUWEHAND A.C. (1998): Antimicrobial components from lactic acid bacteria. In: SALMINEN S., VON WRIGHT X. (Eds.): Lactic Acid Bacteria: Microbiology and Functional Aspects. Marcel Dekker, Inc., New York, 139–159.

PITT J.I., HOCKING A.D. (1985): Spoilage of stored, processed and preserved foods. In: Fungi and Food Spoilage. Acad. Press, North Ryde: 384–395.

ROY U., BATISH V.K., GROVER S., NEELAKANTAN S. (1996): Production of antifungal substance by *Lactobacillus lactis* subsp. *lactis* CHD-28.3. Int. J. Food Microbiol., **32**: 27–34.

STILES J. (1999): The Antifungal Activity of Lactobacilli. [Dissertation Thesis.] VŠCHT Praha.

STILES J., PLOCKOVÁ M., TOTH V., CHUMCHALOVÁ J. (1999): Inhibition of *Fusarium* sp. DMF 0101 by *Lactobacillus* strains grown in MRS and Elliker broths. Adv. Food Sci., **21**: 117–121.

SUZUKI I., NOMURA M., MORICHI T. (1991): Isolation of lactic acid bacteria which suppress mold growth and show antifungal action. Milchwissenschaft, **46**: 635–639.

VANDENBERG P.A., KING S.W. (1988): Process for producing novel yeast and mold inhibiting products. Eur. Patent Appl. 0 302 300.

VEDAMUTHU E.R. (1991): The yoghurt story – past, present and future. Part IV. Dairy, Food Environ. Sanit., **11**: 513–514.

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Souhrn

PLOCKOVÁ M., STILES J., CHUMCHALOVÁ J., HALFAROVÁ R. (2001): **Kontrola růstu plísní kmeny** *Lactobacillus rhamnosus* VT1 **and** *Lactobacillus reuteri* CCM 3625 **sledovaná metodou mléčných agarových ploten**. Czech J. Food Sci., **19**: 46–50.

Schopnost potlačovat růst plísní v mléčném prostředí byla sledována u kmenů *Lactobacillus rhamnosus* VT1 a *Lactobacillus reuteri* CCM 3625 metodou mléčných agarových ploten. Vyšší antifungální aktivitu vykazovaly aktivně rostoucí buňky obou laktobacilů ve srovnání se supernatanty získanými po kultivaci kmenů v MRS bujonu, které obsahovaly naprodukované metabolity. Bylo prokázáno, že inhibice růstu plísní kmenem *Lactobacillus reuteri* CCM 3625 je spojena s produkcí směsi kyselin: mléčné (0,9 % hm.), octové (0,2 % hm.) a jantarové (0,2 % hm.). V případě kmene *Lactobacillus rhamnosus* VT1 inhibici růstu plísní pravděpodobně způsobuje kyselina mléčná (1,2 % hm.) společně s dalšími antifungálně aktivními metabolity nebílkovinné a nesacharidické povahy.

Klíčová slova: *Lactobacillus*; mléčné agarové plotny; kontrola růstu plísní; antifungální metabolit

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