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Inhibition of mould growth and spore production by *Lactobacillus acidophilus* CH5 metabolites

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SUMMARY. In this study antifungal activity of the supernatant containing acidocin CH5 (SCA) after cultivation of *Lactobacillus acidophilus* CH5 in the MRS medium was investigated. SCA caused a suppression of the mycelium growth and a lack of spore formation in 4 out of 4 mould strains belonging to the genera *Penicillium* (2), *Mucor* (1) and *Fusarium* (1).

The first experiment was done by an agar assay using various percentages (v/v) of SCA. The growth of the mould strains was suppressed on GKCH agar plates and on slide cultures. Observations were taken daily both visually and microscopically. *Fusarium* sp. DMF 0101 was found to be the most sensitive to SCA (40 $AU.ml^{-1}$). The inhibition of the mould growth was increased when higher percentages (v/v) of SCA were used.

Interesting results were obtained by the use of supernatants having two different bacteriocin titres and percentages (v/v) of each SCA. This second experiment was done using the same method except that agar medium with malt extract was used. However, the inhibition of the mould growth was increased when higher percentages (v/v) of SCA were used, but not in the case of increasing the total amount of AU per ml of medium.

These results led to the conclusion that the antifungal effect of SCA is not only due to acidocin CH5, but also may be caused by another fermentation product. The antifungal activity of such metabolite was found to be insensitive to both trypsin and pepsin.

Contamination of fermented dairy products such as acidophilus milk, yoghurt and cheeses with undesirable yeasts and moulds is a serious problem [1,2]. Many fungal genera including *Penicillium* spp., *Aspergillus versicolor*, *Aspergillus flavus, Aspergillus glaucus, Cladosporium* spp., *Alternaria* spp., *Geotrichum candidum, Mucor* spp., *Rhizopus* spp. and *Fusarium* spp. were found to be toxic to biological systems [3]. Strains of the genera *Aspergillus* and *Penicillium* were the most frequent mycotoxin producers [4,5].

Interactions between microorganisms grown together in a niche may change the availability of nutrients or result in the production of volatile

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and/or non volatile products. These may stimulate, inhibit, or have no influence on the growth of fungi or on the production and accumulation of a mycotoxin [6,7]. Thus, the interaction between mould contaminants and lactic acid bacteria involved in fermented dairy products needs to be studied.

The antibacterial effects of lactic acid bacteria associated with metabolites such as lactic and acetic acids, hydrogen peroxide [8] and bacteriocins [9,10] were well documented. Less has been published on the antifungal characteristics of lactic acid bacteria [7,11-14]. In our preliminary study, the antifungal activity of neutralized and heat-treated supernatant after the cultivation of *Lactobacillus acidophilus* CH5 on selected strains of yeasts and moulds of the genera *Candida, Kluyveromyces, Penicillium, Fusarium, Cladosporium, Alternaria* and *Geotrichum* was observed [15].

The study was done to evaluate the antifungal activity of *Lactobacillus acidophilus* CH5 cell-free supernatant against the selected mould strains from the genera associated with cheese contamination and to determine whether the antifungal activity might be caused by acidocin CH5 or by some another proteinaceous or non-proteinaceous metabolite.

Material and methods

I. Microorganisms

1. Bacterial strains

Producer of acidocin CH5, *Lactobacillus acidophilus* CH5, was isolated from the dairy starter (Christian Hansen's Laboratory, Denmark).

The indicator strain *Lactobacillus delbrueckii* subsp. *lactis* LTI30 was isolated from the intestinal tract of a child at the Department of Milk and Fat Technology, Institute of Chemical Technology, Prague, Czech Republic.

Both *Lactobacillus* strains were subcultured once a week in MRS broth (Oxoid) using an inoculum size of 1 %, at 37 °C for 16 h.

2. Mould strains

Following strains of moulds were tested:

- *Penicillium* sp. DMF 0006,
- *Penicillium hirsutum* DMF 0001,
- *Mucor* sp. DMF 0501,
- *Fusarium* sp. DMF 0101.

The undesirable strains were isolated from spoiled cheeses at the Department of Milk and Fat Technology. All strains of moulds were subcultured monthly using GKCH agar (agar with glucose, yeast extract and chloramphenicol, Milcom, Czech Republic) slants. Incubation was done at room temperature for 5 days.

For the assessments of antifungal activity, the suspensions of mould strains from the surface of agar slants were washed out with 5 ml of sterile saline containing 0.1 % (v/v) Tween 80 by brushing the surface of the slant with a sterile loop.

II. Methods

Preparation of crude supernatant containing acidocin CH5

Crude supernatant containing acidocin CH5 (SCA) was prepared in the following manner: after a 16 h incubation of *Lactobacillus acidophilus* CH5 (using 1 % inocula) at 37 °C in freshly prepared MRS broth, the pH of the culture was adjusted (with NaOH) to 6.5. The culture was centrifuged at 4 °C for 10 min (4 000 rpm) and the cell biomass was removed. The cell-free supernatant was heated in a water bath for 15 min to inactivate the potentially present unstable antimicrobial substances. After the heat treatment, the supernatant was allowed to cooldown to room temperature and then placed in a freezer for storage at -22 °C.

Acidocin CH5 titer evaluation

In order to measure the amount of activity units present in this SCA, the „Spot-on-lawn" method was used. The indicator strain *Lactobacillus delbrueckii* subsp. *lactis* LTI30 was subcultured before each use in MRS broth (37 °C, 16 h, 1 % inoculum). 70 µl of the indicator organism suspension was mixed with 7 ml of molten MRS medium containing 0.75 % agar (Oxoid) and poured into a Petri dish (∅ 9 cm) with 10 ml of already solidified MRS medium containing 1.0 % agar (Oxoid). Then 20 µl of various dilutions of SCA were applied by stabbing. Incubation of the plate was done at 37 °C overnight. The second day a clear zone of inhibition was indicated. One activity unit (AU) was defined as the reciprocal of the highest dilution yielding a clear zone of inhibition on the indicator lawn [23]. The bacteriocin titer (BT) was calculated using the formula: $BT\ (AU.ml^{-1}) = 2^x.1000/V$, where x is the number of the last dilution causing inhibition and V is the volume of the used supernatant in µl [17].

Assessment of antifungal activity

1. Agar assay

Assessment of antifungal activity was done by agar assay using visual observations of the inhibitions of mould growth (*Penicillium* sp. DMF 0006, *Penicillium hirsutum* DMF 0001, *Mucor* sp. DMF 0501 and *Fusarium* sp. DMF 0101). Agar plates were first prepared by mixing the liquid GKCH agar with various concentrations of SCA (0, 1, 5, 10, 25 and 50 % v/v), containing 400 AU.ml^{-1}. Then the spore suspension (0.2 ml) of each mould strain was inoculated onto the surface of the agar plates. The plates were observed daily (in daylight and at room temperature) for 8 days of cultivation. Changes of mycelium colour appearance, and growth intensity were noted.

To evaluate whether the antifungal activity is caused by acidocin CH5 alone or by another substance present in the cell-free supernatant, another experiment was required. The most sensitive mould strains (*Penicillium* sp. DMF 0006, *Mucor* sp. DMF 0501 and *Fusarium* sp. DMF 0101), two different supernatants of BT = 50 AU.ml^{-1} and 500 AU.ml^{-1}, two different volumes of each SCA (25 and 50 % v/v) and Malt extract agar (Oxoid) for cultivation, were used.

2. Slide cultures

In the first experiment, *Penicillium* sp. DMF 0006, *Penicillium hirsutum* DMF 0001, *Mucor* sp. DMF 0501 and *Fusarium* sp. DMF 0101 slide cultures were prepared by placing a drop of liquid GKCH agar on a glass microscope slide, and subsequently mixed with SCA (0 % and 50 % v/v, respectively). The slides were then inoculated by mould suspensions and incubated on a sterile plate in the same manner as the agar plates mentioned above. During 8 days of cultivation microscopic morphological changes were observed daily.

3. Effects of proteolytic enzymes

Solutions of trypsin and pepsin (Sigma) were prepared in the phosphate buffer at optimal pH values of 5.8. Sets of SCA (BT = 400 AU.ml^{-1}) were treated with enzymes (1 mg.ml^{-1}) and incubated at 37 °C for 2 h. Agar plates (∅ 7 cm) were prepared by mixing liquid GKCH agar with treated SCA (50 % v/v) and suspensions (0.1 ml) of the two most sensitive strains (*Penicillium* sp. DMF 0006 and *Fusarium* sp. DMF 0101) were (after solidification of the agar) inoculated onto the surface of the plates. The plates were incubated in daylight at room temperature and observed daily.

Results and discussion

Previously, the antifungal activity of various lactic acid bacteria, mostly belonging to the genera *Lactococcus* and *Lactobacillus*, isolated from food products such as milk products, vegetables, fruits [14], Cheddar cheese and raw buffalo milk [12], effective especially against penicillia [14,16] and aspergilli [7,12,13], was tested. In the present work, we studied the antifungal potential of *Lactobacillus acidophilus* CH5 - the strain isolated from commercial Chr. Hansen's starter producing bacteriocin acidocin CH5 which was able to inhibit a broad spectrum of bacteria [17].

Macro- and microscopic observations of the tested moulds are presented in Table 1. All tests and observations were done at least twice.

Visual observations clearly showed significant differences between the control plate (without SCA) and the plate with 25 and 50 % (v/v) of SCA regarding the suppression of mould growth. All tested moulds showed an inhibition of growth to a certain degree. The growth of *Penicillium* sp. DMF 0006 and *Fusarium* sp. DMF 0101 were suppressed most strongly. Similar results were obtained by Suzuki at al. [11], for *Fusarium* line IAM 5008 and several tested strains of *Penicillium* sp.

Between *Penicillium hirsutum* DMF 0001 and *Penicillium* sp. DMF 0006 the most important difference could be observed by microscope. *Penicillium* sp. DMF 0006 had smaller and thinner branches with smaller conidia and grew very rapidly in contrast to *Penicillium hirsutum* DMF 0001 which had a very big chain of conidia, and its growth rate was very slow. These morphological structures were intensively changed in comparison with control without addition of SCA. In both strains a significant difference could be observed between the 25 % (v/v) and the 50 % dish from the 3rd day of cultivation.

The strong inhibition was proved for *Fusarium* sp. DMF 0101, where the mycelium color and the morphological appearance differences between the agar surface colonies of mould treated with SCA and untreated ones, were the most evident. On the 3rd day, the mould growth on the plate containing 50 % (v/v) solution of SCA was very strongly suppressed in comparison with the other concentrations which grew very rapidly. Finally, mould growth on this plate stopped from the 5th day, while the others continued.

Even in the case of fast growing mould *Mucor* sp. DMF 0501, the mould on the plate with 50 % (v/v) SCA only began to grow as late as from the 4th day of cultivation. Its color was white-grey in contrast to the other plates which were black from the beginning, indicating the delay in spore

TABLE 1. Inhibition periods of mould growth and vegetative spore production by SCA (BT = 400 AU.ml^{-1}).
TABULKA 1. Časový průběh inhibice růstu plísní a produkce vegetativních spor působením SCA (BT = 400 AU.ml^{-1}).

Growth period[1]	GKCH agar with SCA[2] [% v/v]					Slide cultures[3] [% v/v]	
	0	5	10	25	50	0	50
Penicillium sp. DMF 0006							
1 day[4]	+1	0	0	0	0	$+^0$	$+^1$
2 days	+2	0	0	+	++	$+^0$	$+^1$
3 days	+3	0	0	0	++	nd	nd
8 days	+3	0	0	+	+++	$+^0$	$+^2$
Fusarium sp. DMF 0101							
1 day	-	-	-	-	-	$+^0$	$+^1$
2 days	+1	0	0	+	+	$+^0$	$+^1$
3 days	+1	0	+	++	+++	nd	nd
8 days	+3	0	+	++	+++	$+^0$	$+^2$
Mucor sp. DMF 0501							
1 day	+1	+	+	-	-	$+^0$	$+^1$
2 days	+2	0	0	++	+++	$+^0$	$+^1$
3 days	+3	0	0	++	+++	nd	nd
8 days	+3	0	0	0	++	$+^0$	$+^1$
Penicillium hirsutum DMF 0001							
1 day	-	-	-	-	-	$+^0$	$+^1$
2 days	+1	0	+	++	++	$+^0$	$+^1$
3 days	+1	0	0	++	++	nd	nd
8 days	+3	0	0	+	++	$+^0$	$+^2$

LEGEND: - - no mycelial masses presented, +1 - visible mycelial masses, +2 - lawn on mycelial masses, +3 - vegetative spores present, change in colour and appearance of mould colony, 0 - no suppression, the same growth as control, + - weak suppression compared with control, ++ - strong suppression compared with control, +++ - very strong suppression compared with control, $+^0$ - morphology of hyphae, specialized hyphae and vegetative spores typical for the particular mould strains observed, $+^1$ - weak suppression (less mass of hyphae, less vegetative spores), $+^2$ - strong suppression (changes in the size and morphology of hyphae, specialized hyphae and vegetative spores), nd - not detected.

1 - růstová fáze, 2 - GKCH agar s přídavkem SCA, 3 - sklíčkové kultury, 4 - den.
LEGENDA: - - mycelium nebylo přítomno, +1 - mycelium pozorovatelné, +2 - hustý myceliální porost pokrývající celý povrch misky, +3 - vegetativní spory přítomny, změna v barvě a vzhledu kolonie plísně, 0 - neprobíhá potlačení růstu, růst totožný s kontrolou, + - ve srovnání s kontrolou slabé potlačení růstu, ++ - ve srovnání s kontrolou silné potlačení růstu, +++ - ve srovnání s kontrolou velmi silné potlačení růstu, $+^0$ - morfologie hyf, specializované hyfy a vegetativní spory typické pro jednotlivé pozorované kmeny plísní, $+^1$ - slabé potlačení (menší množství hyf, méně vegetativních spor), $+^2$ - silné potlačení (změny ve velikosti a morfologii hyf, specializovaných hyf a vegetativních spor), nd - neměřeno, SCA - supernatant obsahující acidocin CH5.

production. The same was acknowledged by use of slide cultures where the lack of many sporangiophores with sporangiae in the 50 % (v/v) slide could be also observed.

The results from visual observation of appearance of mould colonies on Petri dishes and microscopic observation of mould structures on slide cultures were similar.

In our previous study [15] the antifungal activity was tested by use of the „Spot-on-lawn“ agar diffusion assay, but the method was not found to be

TABLE 2. Inhibition periods of mould growth by use of two different SCA on Malt extract agar.
TABULKA 2. Časový průběh inhibice růstu plísní na Malt extrakt agaru supernatanty s různým obsahem acidocinu CH5.

Growth period[1]	Control[2] of BT = 0 AU.ml^{-1} [% v/v]	SCA[3] of BT = 50 AU.ml^{-1} [% v/v]		SCA[4] of BT = 500 AU.ml^{-1} [% v/v]	
	0	25	50	25	50
Penicillium sp. DMF 0006					
1 day[5]	+1	0	0	0	0
2 days	+2	0	0	0	0
3 days	+3	0	+	0	+++
8 days	+3	0	++	0	+++
Fusarium sp. DMF 0101					
1 day	-	-	-	-	-
2 days	+1	0	0	0	0
3 days	+1	0	++	++	+++
8 days	+3	0	+++	++	+++
Mucor sp. DMF 0101					
1 day	+1	0	0	0	0
2 days	+2	0	0	0	0
3 days	+3	+	++	+	++
8 days	+3	+	++	+	++

LEGEND: - - no mycelial masses presented, +1 - visible mycelial masses, +2 - lawn on mycelial masses, +3 - vegetative spores present, change in color and appearance of mould colony, 0 - no suppression, the same growth as control, + - weak suppression compared with control, ++ - strong suppression compared with control, +++ - very strong suppression compared with control.

1 - růstová fáze, 2 - kontrola BT = 0 AU.ml^{-1}, 3 - SCA BT = 50 AU.ml^{-1}, 4 - SCA BT = 500 AU.ml^{-1}, 5 - den.

LEGENDA: - - mycelium nebylo přítomno, +1 - mycelium pozorovatelné, +2 - hustý myceliální porost pokrývající celý povrch misky, +3 - vegetativní spory přítomny, změna v barvě a vzhledu kolonie plísně, 0 - neprobíhá potlačení růstu, růst totožný s kontrolou, + - ve srovnání s kontrolou slabé potlačení růstu, ++ - ve srovnání s kontrolou silné potlačení růstu, +++ - ve srovnání s kontrolou velmi silné potlačení růstu, nd - neměřeno, SCA - supernatant obsahující acidocin CH5, BT - bakteriocinový titr.

sensitive enough for our purposes. For this reason, the present work method mentioned above was developed and used with excellent results.

An overall analysis of the results leads to a conclusion that SCA possesses antifungal activity on four tested mould strains. With regard to the results obtained in previous experiments, the antifungal activity of *Lactobacillus acidophilus* CH5 supernatant was repeatedly proved for both *Penicillium* strains and newly discovered for *Mucor* sp. and *Fusarium* sp.

The results obtained by the use of supernatants having two different bacteriocin titres and volume percentages of each SCA are presented in Table 2. The observations show that inhibition of mould growth was increased when higher volume percentages of SCA was used, but not in the case of increasing the total amount of AU per ml of medium.

The effect of the treatment of SCA with proteolytic enzymes trypsin and pepsin is shown in Table 3. The antifungal activity of SCA was found to be not sensitive to both trypsin and pepsin.

According to the results and hypotheses presented in literature, opinions on the antifungal action of lactic acid bacteria are very different. The antifungal activity used to be associated with both - actively growing and metabolizing cells of lactic acid bacteria [11], and metabolites of lactic acid bacte-

TABLE 3. The effect of proteolytic enzymes on the antifungal potential of cell-free supernatant of acidocin CH5.
TABULKA 3. Účinek proteolytických enzymů na antifungální aktivitu bezbuněčného supernatantu obsahujícího acidocin CH5.

Growth period[1]	*Penicillium* sp. DMF 0006				*Fusarium* sp. DMF 0101			
	control[2]	SCA[3] + trypsin	SCA[4] + pepsin	SCA[5] untreated	control	SCA + trypsin	SCA + pepsin	SCA untreated
1 day[6]	-	-	-	-	-	-	-	-
2 days	+2	+++	+++	++	+1	+++	++	+++
3 days	+3	+++	+++	++	+2	+++	+++	+++

LEGEND: - - no mycelial masses presented, +1 - visible mycelial masses, +2 - lawn on mycelial masses, +3 - vegetative spores present, change in color and appearance of mould colony, 0 - no suppression, the same growth as control, + - weak suppression compared with control, ++ - strong suppression compared with control, +++ - very strong suppression compared with control.

1 - růstová fáze, 2 - kontrola, 3 - SCA + trypsin, 4 - SCA + pepsin, 5 - neošetřený supernatant, 6 - den.
LEGENDA: - - mycelium nebylo přítomno, +1 - mycelium pozorovatelné, +2 - hustý myceliální porost pokrývající celý povrch misky, +3 - vegetativní spory přítomny, změna v barvě a vzhledu kolonie plísně, 0 - neprobíhá potlačení růstu, růst totožný s kontrolou, + - ve srovnání s kontrolou slabé potlačení růstu, ++ - ve srovnání s kontrolou silné potlačení růstu, +++ - ve srovnání s kontrolou velmi silné potlačení růstu, SCA - supernatant obsahující acidocin CH5.

ria. According to Batish [18], the antifungal activity of lactic acid bacteria appeared to be related to lactic and acetic acids; and according to Warminska - Radyko [19], to propionic acid produced by propionic acid bacteria. Vandenbergh [16] found the compound produced by *Lactobacillus casei* var. *rhamnosus* and antifungally active against *Penicillium oxalicum* to be a polar substance of molecular size of less than 1000 daltons but it has not proteinaceous or lipidic character. Gourama [14] discovered inhibitory activity in two *Lactobacillus casei* cell-free supernatants unrelated to the production of lactic acid and hydrogen peroxide, sensitive to proteolytic enzymes and high temperature (100 °C). Several authors published the antifungal activity of *Lactobacillus acidophilus* strains against *Candida albicans* [20], against growth and aflatoxin production of *Aspergillus flavus* [13,21] or *Aspergillus fumigatus, Aspergillus parasiticus* and *Rhizopus stolonifer*.

In this case, the antifungal activity of *Lactobacillus acidophilus* CH5 was related to cell-free supernatant treated by heat (99 °C/15 min) to avoid the activity of thermounstable and volatile compounds, including hydrogen peroxide. It was also neutralized to avoid unwanted organic acid action.

Further more, it was found that the antifungal activity did not depend on the proteinaceous acidocin CH5 concentration, but its efficacy depended on the concentration of the added supernatant. The results of the proteolytic enzymes action on the antifungal substance stimulated our intent to study this aspect further using the following groups of enzymes: proteolytic, saccharolytic and lipolytic.

It is interesting that the antifungal substance exhibited the broad inhibitory spectrum against yeasts and moulds including the genera *Penicillium, Mucor, Fusarium, Cladosporium, Alternaria, Geotrichum, Candida* and *Kluyveromyces*. This is similar to the broad spectrum of natamycin-metabolite of *Streptomyces natalensis* [22], used for prevention of mould growth on cheese surface.

Conclusions

Regarding the previously published results, it can be concluded that the neutralized and heat treated supernatant after cultivation of *Lactobacillus acidophilus* CH5 in MRS broth exhibited the broad antifungal spectrum against 3 out of 4 tested yeast strains, and 7 of 14 mould strains. The responsibility for antifungal activity is potentially not only due to acidocin CH5. Therefore, the challenge for the future is to discover the agent

responsible for the antifungal activity. It is interesting that the strain producing antibacterial active acidocin CH5, produces also an antifungal metabolite.

Lactic acid bacteria, particularly *Lactobacillus acidophilus* CH5, which possesses antifungal activity, have promising potential for its application in dairy industry as well as in other branches of the food industry.

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List of abbreviations

SCA	supernatant containing acidocin CH5
BT	bacteriocin titer
$AU.ml^{-1}$	activity units per mililiter

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Inhibice růstu plísní a jejich sporulace metabolity kmene *Lactobacillus acidophilus* CH5

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SOUHRN. V této práci byla prověřována antifungální aktivita supernatantu obsahujícího acidocin CH5 (SCA) po kultivaci kmene *Lactobacillus acidophilus* CH5 v MRS bujonu. SCA potlačil růst mycelia a tvorbu spor u 4 testovaných kmenů plísní rodu *Penicillium, Mucor* a *Fusarium*.

V prvním pokusu byla použita agarová metoda s různými koncentracemi (v/v) SCA. Růst kmenů plísní byl potlačen na GKCH agarových miskách a na sklíčkových kulturách. Jak visuální, tak mikroskopické pozorování bylo prováděno denně. Bylo zjištěno, že *Fusarium* sp. DMF 0101 je nejcitlivější k účinku SCA (40 $AU.ml^{-1}$). Podle očekávání byla inhibice růstu kmenů plísní zvýšena, jestliže se zvýšila koncentrace (v/v) SCA.

Zajímavé výsledky byly získány použitím dvou různých SCA, mající rozdílný bakteriocinový titr a použité v různých koncentracích (v/v). V tomto případě byl pro agarovou metodu použit agar s maltosovým extraktem. Inhibice růstu plísní byla zvýšena, jestliže se zvýšila koncentrace (v/v) SCA, a ne v případě zvýšení celkového obsahu AU na jeden ml media.

Tyto výsledky vedou k závěru, že antifungální aktivita SCA není způsobena pouze aci-

docinem CH5, ale může být zapříčiněna jiným produktem fermentace. Bylo také zjištěno, že antifungální aktivita tohoto metabolitu není citlivá k proteolytickým enzymům pepsinu a trypsinu.