Antifungal activity and aflatoxin binding ability of *Lactobacillus* species isolated from lamb and goatling stomach mucus

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Summary

Eight isolates from lamb and goatling stomach mucus belonging to *Lactobacillus reuteri*, *Lb. plantarum, Lb. mucosae*, *Lb. murinus* and a dairy strain *Lb. reuteri* CCM 3625 were characterized for their antifungal activity. The ability of five selected strains to bind aflatoxin B_1 was determined in vitro by high-performance liquid chromatography. Impact of pH, inactivated *Escherichia coli* and additives, namely, phenylalanine, tyrosine and α-ketoglutarate, on the antifungal activity of the strains was evaluated. Production of phenyllactic acid and 4-hydroxyphenyllactic acid was measured in the cell-free supernatant with optimized composition. All tested strains showed antifungal activity against tested fungi (7.4–43.0 % of growth inhibition). The antifungal activity was enhanced at pH 4.0 in most cases. Supplementation of the medium by phenylalanine, tyrosine and α-ketoglutarate increased the antifungal activity against *Aspergillus flavus* and *Rhizopus oryzae*. The best producer of phenyllactic acid was *Lb. plantarum* KG4 (0.7400 ± 0.0217 $mmol \cdot l^{-1}$). The tested strains bound 10.8–66.7 % of aflatoxin B_1 present in the sample. A combination of both the antifungal activity and activity to bind aflatoxin B_1 indicates the potential of the strains to eliminate toxins and fungal contamination of food and feed as well as other substances like heavy metals in practice.

Keywords

bioconservation; *Lactobacillus*; phenyllactic acid; fungi; aflatoxin B_1; high-performance liquid chromatography

Spoilage of food and feed due to fungal growth and synthesis of mycotoxins is a frequent problem having a negative impact on health, economy and food security [1]. Aflatoxins are produced by some strains of *Aspergillus* genus, having detrimental health effects including mutagenic and carcinogenic effects in humans and animals [2]. Aflatoxin AFB_1 is the most prevalent mycotoxin in the food and it is toxic for man and animals [3]. It was estimated that more than 4.5 billion people are chronically exposed to aflatoxins [4]. Various methods are applied to prevent the fungal spoilage or to mitigate mycotoxins. One promising approach is based on the use of lactic acid bacteria (LAB). A number of reports documenting the antifungal activity of LAB has increased during the last decade [5]. The antifungal metabolites of LAB identified to date encompass a diverse group of compounds including various organic acids, cyclic dipeptides, fatty acids, and proteinaceous compounds [6]. Lactic acid and acetic acid were found to inhibit the undesirable food microflora [7]. Besides lactic acid and acetic acid, phenyllactic acid showed reasonable antifungal effects [8, 9]. Multiple compounds may act in synergistic action and thus contribute to the broad spectrum of antifungal activity. Antifungal metabolite-producing isolates of LAB have been successfully applied in the preservation of a variety of foods [10–12]. The aerobic quality of silage can be also improved by the addition of *Lb. buchneri* strains [13]. Concentrated cell-free supernatant (CFS) from *Lb. plantarum* 16 was found to be more effective as common antifungal agents against

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the yeast *Rhodotorula mucilaginosa*, in comparison with sodium benzoate and potassium sorbate at their Food and Drug Administration (FDA) maximum permitted level [14]. CFS of *Lactobacillus plantarum* YML007 was successfully used as preservative on soya beans [15]. Specific strains of LAB were shown to bind non-covalently the potent toxin AFB_1 that contaminates food and feed supplies on a global scale [16–19]. Some authors suggested that aflatoxin binds predominantly to polysaccharides and peptidoglycans of the bacterial cell wall [20, 21]. Hydrophobic interactions play a major role in AFB_1 binding by these bacteria. Electrostatic interactions also have some effect [20, 22]. This attribute is strain-specific. Acid and heat treatment were found to increase the initial binding and adhesion after aqueous washing [22]. Some strains of *Lactobacillus* genus showed good AFB_1 binding [19]. El-Nezami et al. [16] reported aflatoxin binding by *Lb. rhamnosus* GG (up to 81.0 %) and *Lb. rhamnosus* LC705 (up to 82.0 %) with heat-killed bacteria. Relatively good results were obtained by Hernandez-Mendoza et al. [18] with *Lb. reuteri* NRRL14171. Initial binding was almost 60 % (at 0 h) and increased up to 80 % during 12 h incubation at pH 7.2. LAB have a potential not only to bind already produced aflatoxins, but also can inhibit the growth of fungi and consequently inhibit aflatoxin production [23]. Indeed, these facts indicate that evaluation of both aspects of the LAB biological activity are reasonable.

The aim of our study was to investigate antifungal activity and aflatoxin binding potential of new potentially probiotic strains isolated from stomach mucus of lamb, namely, *Lb. reuteri* E and *Lb. mucosae* D, *Lb. murinus* C and, from goatling, *Lb. reuteri* KO5, *Lb. reuteri* KO4b, *Lb. reuteri* KO4m, and *Lb. plantarum* KG1, *Lb. plantarum* KG4. Strains were identified by sequencing of 16S rRNA [24, 25]. Antimicrobial activity of *Lb. reuteri* E, *Lb. mucosae* D and *Lb. murinus* C, and immunomodulation activity of *Lb. reuteri* E in vitro and in vivo in a mouse model were evaluated [26, 27]. Bile tolerance of all strains was also tested [28]. Characterization of these lactobacilli for their antifungal activity and for their ability to bind aflatoxin in in vitro assay should lead to the selection of the biologically most active species useful for biological conservation of food or feed.

MATERIALS AND METHODS

Bacterial strains

Lactobacillus strains used in the work were *Lb. reuteri* E, *Lb. mucosae* D, *Lb. murinus* C isolated from stomach mucus of lamb, *Lb. reuteri* KO5, *Lb. reuteri* KO4b, *Lb. reuteri* KO4m, *Lb. plantarum* KG1, *Lb. plantarum* KG4 isolated from stomach mucus of goatling and *Lb. reuteri* CCM 3625 from Czech Collection of Microorganisms (CCM; Masaryk University, Brno, Czech Republic). Stock cultures were maintained at –70 °C in 15% (v/v) glycerol. Strains were cultured in de Man, Rogosa and Sharpe (MRS) broth (Difco, Sparks, Maryland, USA) at 37 °C for 18 h.

Fungal strains

Strains of *Aspergillus flavus* CCM F-171, *A. niger* CCM F-8004, *Fusarium culmorum* CCM F-21, *F. nivale* CCM F-429, *Mucor racemosus* CCM F-8109, *Penicillium chrysogenum* CCM F-432, *P. purpurogenum* CCM F-472 and *Rhizopus oryzae* CCM F-8284 were selected as common contaminants of food and feed. Fungal strains were cultivated on Sabouraud agar (Difco) at 25 °C until sporulation occurred. Spores were then collected by suspending in saline solution (0.85% NaCl) containing 0.1 % (v/v) Tween 80 (LCHM-Labochem, Bratislava, Slovakia). Spore concentration was determined by counting in Bürker chamber and adjusted to 10^4 spores per millilitre with the saline solution with Tween.

Dual-culture overlay assay.

Dual culture overlay assay was done according to Magnusson et al. [29] with slight modifications. Lactobacilli were cultured in MRS broth overnight at 37 °C and adjusted to optical density at 600 nm A_{600} = 1.8. A sterile paper disk (diameter 12 mm; GE Healthcare, Little Chalfont, United Kingdom) was placed in the center of a plate on the surface of MRS agar and 100 μl of an overnight culture of individual strains were pipetted on paper disk. Plates were incubated at 37 °C for 48 h in anaerobic conditions. Then, the plates with lactobacilli were overlaid with 2 ml of Sabouraud agar (Difco) containing 20 μl of a spore suspension (10^5 spores per millilitre) of fungi and incubated at 25 °C for 2 or 7 days, according to the fungus. The percentage of growth inhibition (I) was calculated according to Eq 1.

$$I = \frac{r_A^2}{r_B^2} \times 100 \quad (1)$$

where r_A is radius of inhibition zone and r_B is radius of Petri dish.

Cell-free supernatant preparation

Lactobacillus cultures were propagated in MRS

broth with 30 g·l^{-1} glucose for 72 h at 37 °C under anaerobic conditions, or in MRS broth supplemented with 0.5 g·l^{-1} phenylalanine (Phe), 0.1 g·l^{-1} tyrosine (Tyr) and 0.2 g·l^{-1} α-ketoglutarate (α-KG) (Sigma-Aldrich, St. Louis, Missouri, USA) [30]. Cells were collected by centrifugation at 4 000 ×*g* for 10 min at 4 °C. Supernatants were filtered through a microfilter (pore size 0.22 μm; Fisher, Hampton, United Kingdom) to remove any residual cells. The cell-free supernatant (CFS) was used immediately or was stored frozen at –20 °C for later use. pH of the supernatants ranged from 3.6 to 3.9.

Co-cultivation of *Lactobacillus* strains with *E. coli*

For stimulation of reuterin production, *Lb. reuteri* strains were co-incubated with inactivated cells of *Escherichia coli* K12. Bacterial culture of *E. coli* K12 was subcultered in Luria-Bertani (LB) broth (Sigma-Aldrich) overnight at 37 °C and adjusted to A_{600} = 0.12. Diluted cell suspension in a Petri dish was then exposed to ultraviolet C (UV-C) light at 30 cm for 30 min. Loss of viability of *E. coli* was confirmed by plating cells onto LB agar (Sigma-Aldrich) and incubating overnight at 37 °C. A volume of 50 ml of MRS supplemented with 250 mmol·l^{-1} glycerol was inoculated with *Lactobacillus* cells (150 mg) and *E. coli* cells (50 mg), and incubated for 72 h at 37 °C anaerobically. CFS was prepared as described above.

Effect of pH on antifungal activity of cell-free supernatant

Sabouraud dextrose agar media (adjusted to pH 3.0, pH 4.0, pH 5.0) containing 10% (v/v) of CFS of each strain were prepared. A volume of 5 μl of spore suspension (10^4 spores per millilitre) was centrally spotted on the plate. Control plates contained Sabouraud dextrose agar containing 10% (v/v) MRS broth. Inoculated plates were cultured at 25 °C for 2–7 days, depending on species, regarding its growth rate. The area of mycelial growth in treated plates (A_t) and control plates (A_c) was determined from the mean perpendicular diameter measurements assuming a circular growth. The percentage of growth inhibition (I) was calculated according to Eq. 2 [31].

$$I = \frac{A_c - A_t}{A_c} \times 100 \quad (2)$$

Aflatoxin binding assay

Five strains were used in this study, namely, *Lb. reuteri* E and *Lb. mucosae* D from lamb, *Lb. reuteri* KO4b, *Lb. plantarum* KG4 from goatling and *Lb. reuteri* CCM 3625, which were maintained by routine subculture on MRS agar (Difco). The strains were cultured in MRS broth prior the binding experiments for 24 h. Aliquot of 1% from subculture were inoculated to 50 ml of MRS broth and incubated for 18 h without shaking at 37 °C. Bacterial cell population was adjusted to 10^9 CFU·ml^{-1} based on A_{600}. Cells were collected by centrifugation (3 200 ×*g*, 10 min, 10 °C) and washed twice with phosphate buffered saline (PBS; Thermo-Fisher Scientific, Waltham, Massachusetts, USA) with pH 7.2. A volume of 1 ml of the re-suspended bacterial suspension were employed to carry out the AFB_1 binding assay.

The in vitro binding assays were performed according to El-Nezami et al. [16] with slight modifications. Briefly, 1 ml of each active culture suspended in PBS was centrifuged (3 200 ×*g*, 10 min, 10 °C). Then, the bacterial pellet was suspended in 1 ml of the working solution of AFB_1 (5 μg·ml^{-1} in PBS, pH 7.2) and incubated at 37 °C for periods of 0 h, 4 h and 24 h. Cells were then collected by centrifugation (4 000 ×*g*, 10 min, 10 °C) and the supernatant fluid containing the residual AFB_1 was collected for further analysis by high-performance liquid chromatography (HPLC). For each strain, a control (bacteria suspended in PBS) and an AFB_1 control (working solution of AFB_1) were also incubated. All strains were analysed by two independent experiments and each experiment was carried out in triplicate.

Preparation of AFB_1 standard solution

The stock standard solution of AFB_1 (AFB_1 produced by *Aspergillus flavus,* ≥98% as measured by thin layer chromatography and HPLC, Sigma-Aldrich) of a concentration of 1.0 mg·ml^{-1} was prepared in acetonitrile (≥99.9 %, Sigma-Aldrich). The working solutions of AFB_1 were prepared in PBS (NaCl 9 g·l^{-1}, $Na_2HPO_4.7H_2O$ 0.726 g·l^{-1}, KH_2PO_4 0.21 g·l^{-1}, pH 7.2) and used for analyses in the concentration of 5 μg·ml^{-1}.

AFB_1 determination

An HPLC system HP 1050 Series (Agilent, Santa Clara, California, USA) equipped with an auto-sampler and a scanning fluorescence detector (Agilent 1100) set at the excitation and emission wavelengths of 365 nm and 425 nm, respectively, was used. The separation was performed on a stainless-steel column Zorbax Eclipse XDB-C18 (Agilent, 150 mm × 4.6 mm, particle size 5 μm), connected to a guard column Zorbax Eclipse XDB-C18 (Agilent, 12.5 mm × 4.6 mm, particle size 5 μm). Acetonitrile with acidified water (2% (v/v) acetic acid 99.9% HPLC grade

(Sigma-Aldrich), Milli Q water DIRECT-Q 3UV (Merck Millipore, Billerica, Massachusetts, USA), at a flow rate of 0.9 ml·min^{-1}, was used as the mobile phase. All analyses were carried out at ambient temperature. The HPLC system was controlled by Agilent ChemStation. The peak of AFB_1 was identified through the corresponding retention time confirmed by the reference standard of AFB_1. The calibration measurements were carried out with AFB_1 standard solutions. Linear response of fluorescence detector was estimated in the range of concentrations 0.0032–6.05 μg·ml^{-1}, which granted $r > 0.999$. The limit of detection (*LOD*) for AFB_1 was 0.0025 μg·ml^{-1}; limit of quantification (*LOQ*) was 0.0032 μg·ml^{-1}. *LOD* and *LOQ* were calculated using equations Eq. 3 and Eq. 4, respectively.

$$LOD = X_0 + 3SD \quad (3)$$

$$LOQ = X_0 + 10SD \quad (4)$$

where X_0 is an average response of blank samples, *SD* is standard deviation for $n = 5$.

Phenyllactic acid and 4-hydroxyphenyllactic acid determination

The HPLC system was equipped with an isocratic pump DeltaChrom SDS 030 (Watrex, Bratislava, Slovakia), a manual injector Rheodyne 7725i (IDEX, Middleboro, Massachusetts, USA) fitted with a 20 µl loop, and a UV detector Applied Biosystems 759A (Applied Biosystems, Foster City, California, USA) set to 210 nm. Data were collected on a Clarity system (Watrex). The analyses were performed isocratically at 0.7 ml·min^{-1}, at 55 °C with a 300 mm × 7.8 mm cation exchange column Aminex HPX-87H (Bio-Rad Laboratories, Hercules, California, USA) equipped with a cation H^+ microguard cartridge (Bio-Rad). Mobile phase was 0.5 mmol·l^{-1} H_2SO_4 prepared by diluting reagent-grade sulfuric acid with water for HPLC, and degassing by sonication. Analytical grade reagents were used as standards (Sigma-Aldrich). The peaks of 3-phenyllactic acid (PLA) and 4-hydroxyphenyllactic acid (4-OHPLA) were identified through the corresponding retention times confirmed by the reference standards of PLA and 4-OHPLA. The calibration measurements were carried out with 4-OHPLA and PLA standard solutions. The linear response of UV detector was estimated in the range of concentrations 0.0920–18.4205 μg·ml^{-1} (4-OHPLA) and 0.1480–18.3362 μg·ml^{-1} (PLA), which granted $r > 0.999$ for both analytes. *LOD* for 4-OHPLA was 0.0281 μg·ml^{-1}; *LOQ* was 0.0850 μg·ml^{-1}. *LOD* for PLA was 0.0455 μg·ml^{-1}; *LOQ* was 0.0850 μg·ml^{-1}. *LOD* and *LOQ* were calculated using equations Eq. 3 and Eq. 4, respectively, where X_0 is an average response of blank samples, *SD* is standard deviation for $n = 7$.

Statistical analysis

All experiments were done in triplicates. Data were statistically analysed by one-way ANOVA with post-hoc Tukey's honestly significant difference (HSD) test and unpaired *t*-test.

RESULTS AND DISCUSSION

Antifungal activity in dual culture overlay assay

Eight animal *Lactobacillus* spp. strains and one collection strain *Lb. reuteri* CCM 3625 were tested for their antifungal activity by dual culture overlay assay against seven fungal species, the common food contaminants. Some of them, like *A. flavus* or *F. culmorum*, are mycotoxin producers. All tested *Lactobacillus* strains exhibited antifungal activity (Fig. 1). The most susceptible fungi were *M. racemosus* with 43.0% (*Lb. reuteri* KO4b) and *R. oryzae* 39.4% (*Lb. reuteri* KO4b) of growth inhibition, measured as the reduction of the area of mycelial growth. The strains with the most potent antifungal activity were *Lb. reuteri* CCM 3625, *Lb. reuteri* KO4b, *Lb. reuteri* KO5, *Lb. plantarum* KG4 and *Lb. mucosae* D. The lowest antifungal activity showed *Lb. murinus* C. The highest growth inhibition of *A. flavus* showed *Lb. mucosae* D (30.9%). Here we observed that the antifungal activity depended on the strain as well as on the fungal species.

Antifungal activity of cell-free supernatant and impact of pH

The antifungal activity of the sole CFS of lactobacilli strains was tested. Antifungal activity was determined in the acidic region of pH (pH 3, pH 4 and pH 5) on Sabouraud agar. The highest inhibition of the fungal growth was observed at pH 4 for majority of the strains and fungi (Tab. 1). Significant difference between each pH was found in *Lb. murinus* C, *Lb. reuteri* KO4m ($p < 0.05$), *Lb. reuteri* KO5, *Lb. reuteri* E, *Lb. plantarum* KG1 and *Lb. plantarum* KG4 ($p < 0.01$). At the optimum pH 4, the strains *Lb. reuteri* E and *Lb. reuteri* CCM 3625 were the most active against fungal species. Cortés-Zavaleta et al. [31], using the same method, observed inhibition of *A. flavus* in the range of 2.7–35.4% . Our strains inhibited *A. flavus* even more effectively, i.e. in the range of 21.6–41.5%. Three mechanisms may explain the antimicrobial efficiency of LAB, namely, prosuc-

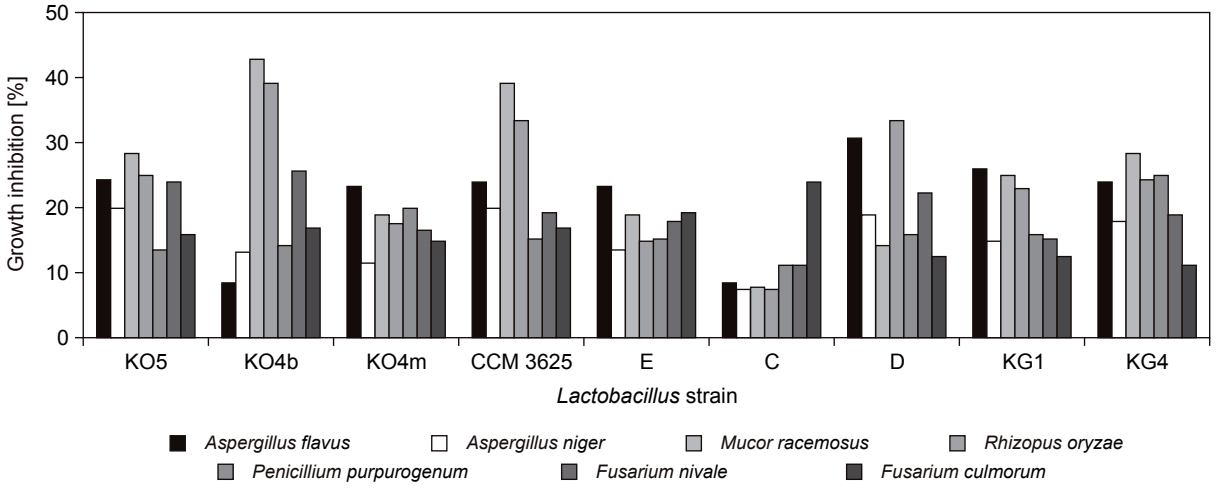

Fig. 1. Antifungal activity of lactobacilli strains against fungal species.

Antifungal activity was evaluated as percentage of growth inhibition calculated according to Eq. 1. compared to control plate without pre-cultivated lactobacilli.
KO5 – *Lb. reuteri* KO5, KO4b – *Lb. reuteri* KO4b, KO4m – *Lb. reuteri* KO4m, CCM 3625 – *Lb. reuteri* CCM 3625, E – *Lb. reuteri* E, C – *Lb. murinus* C, D – *Lb. mucosae* D, KG1 – *Lb. plantarum* KG1, KG4 – *Lb. plantarum* KG4.

Tab. 1. Antifungal activity of cell-free supernatants of lactobacilli.

Strain	Growth inhibition of fungal species [%]					
	Conditions	*R. oryzae*	*M. racemosus*	*A. niger*	*A. flavus*	*P. chrysogenum*
Lb. reuteri KO5	pH 3	10.7	3.8	0.0	26.4	14.2
	pH 4	33.9	52.3	38.2	36.4	50.7
	pH 5	23.9	37.0	27.6	10.1	35.6
Lb. reuteri KO4b	pH 3	45.0	17.0	0.0	15.0	23.0
	pH 4	42.9	59.6	16.9	34.8	59.9
	pH 5	16.9	40.3	32.6	12.5	32.9
Lb. reuteri KO4m	pH 3	7.0	23.5	7.4	26.7	20.2
	pH 4	3.0	42.4	21.5	24.8	49.9
	pH 5	29.5	31.4	12.6	15.0	26.4
Lb. reuteri CCM 3625	pH 3	48.8	21.8	0.0	23.8	0.0
	pH 4	41.3	53.4	21.5	27.0	61.6
	pH 5	42.9	47.4	6.9	25.3	31.7
Lb. reuteri E	pH 3	40.5	5.7	0.0	17.2	0.0
	pH 4	42.9	55.3	28.6	41.5	62.8
	pH 5	15.9	15.0	5.2	0.9	0.0
Lb. murinus C	pH 3	61.0	44.8	3.0	26.8	20.0
	pH 4	45.3	49.4	25.3	32.6	43.0
	pH 5	6.0	19.5	12.0	10.6	11.1
Lb. mucosae D	pH 3	54.4	44.8	7.0	28.0	40.0
	pH 4	41.9	46.8	10.2	21.6	23.1
	pH 5	14.7	36.3	19.9	46.1	24.9
Lb. plantarum KG1	pH 3	7.8	10.3	12.1	22.6	15.3
	pH 4	35.7	43.2	27.3	34.6	27.3
	pH 5	11.9	34.8	15.5	4.4	22.7
Lb. plantarum KG4	pH 3	49.9	33.0	6.0	23.0	36.0
	pH 4	54.3	53.8	29.8	34.6	54.7
	pH 5	5.0	16.5	5.2	5.8	31.4

Sabouraud agar was adjusted to pH 3.0, pH 4.0 or pH 5.0 and supplemented with 10% (v/v) cell-free supernatant.

tion of organic acid, competition for nutrients and production of antagonistic compounds (e.g. bacteriocins) [29]. Higher inhibition rate determined by this method, in comparison to dual culture overlay assay, could be explained by lower pH of the culture media. Usage of CFS instead of live bacteria can overcome the animal origin of our strains. Acidic pH is known to be critical for antifungal activity of many LAB, because the majority of antifungal metabolites of LAB are organic acids and hydroxyl fatty acids. Adjusting pH of CFS to 6.5 or higher reduced its antifungal activity [5, 31].

Impact of culture conditions on antifungal activity of cell-free supernatant

SCHAEFER et al. [32] documented an induction of reuterin production by the incubation of *L. reuteri* with inactivated *E. coli*. Reuterin production by *L. reuteri* E and *L. reuteri* KO5 was previously confirmed [33]. Therefore, our *L. reuteri* strains were cultivated in MRS broth supplemented with 250 mmol·l^{-1} glycerol and with the cells of *E. coli* K12 inactivated by UV irradiation. Antifungal activity was evaluated on Sabouraud agar adjusted to pH 4. Activity of the individual strains was not significant, with the exception of the activity against *R. oryzae* ($p < 0.01$).

Impact of the medium composition on antifungal activity was evaluated, the results obtained are presented in Tab. 2. Generally, a significant difference in antifungal activity with and without the aminoacid supplementation was detected. In the case of individual strains of fungi, significant differences in terms of an increased inhibition between MRS and supplemented MRS against *R. oryzae* and *A. flavus* were observed. The antifungal activity of CFS from supplemented MRS against *M. racemosus* was significantly lower than of CFS from the standard MRS with 3% glucose. Production of PLA and 4-OHPLA was evaluated by HPLC. All the strains, except *Lb. reuteri* KO4b, produced PLA and 4-OHPLA (Fig. 2).

Tab. 2. Antifungal activity of cell-free supernatant of lactobacilli in various cultivation conditions.

Strain	Growth inhibition of fungal species [%]					
	Conditions	*R. oryzae*	*M. racemosus*	*A. niger*	*A. flavus*	*P. chrysogenum*
Lb. reuteri KO5	MRS	33.9	52.3	38.2	36.4	50.7
	sMRS	66.7	40.8	17.8	57.3	57.7
	E. coli	63.2	53.1	5.2	19.6	32.5
Lb. reuteri KO4b	MRS	42.9	59.6	16.9	34.8	59.9
	sMRS	69.4	43.4	20.8	44.1	53.4
	E. coli	63.0	48.1	3.6	21.7	37.8
Lb. reuteri KO4m	MRS	3.0	42.4	21.5	24.8	49,9
	sMRS	58.7	43.0	20.1	34.0	37.2
	E. coli	60.0	45.9	3.0	27.0	48.3
Lb. reuteri CCM 3625	MRS	41.3	53.4	21.5	27.0	61.6
	sMRS	70.7	47.4	26.7	47.5	59.1
	E. coli	78.6	52.9	26.6	33.3	50.8
Lb. reuteri E	MRS	42.9	55.3	28.6	41.5	62.8
	sMRS	77.9	48.6	24.6	48.0	59.1
	E. coli	89.3	69.5	32.8	43.0	56.8
Lb. murinus C	MRS	45.3	49.4	25.3	32.6	43.0
	sMRS	62.4	43.0	7.5	37.3	40.6
Lb. mucosae D	MRS	41.9	46.8	10.2	21.6	23.1
	sMRS	63.1	41.2	12.3	39.9	53.9
Lb. plantarum KG1	MRS	35.7	43.2	27.3	34.6	27.3
	sMRS	65.3	46.6	23.8	48.0	61.2
Lb. plantarum KG4	MRS	54.3	53.8	29.8	34.6	54.7
	sMRS	72.6	45.0	16.2	41.5	50.3

Sabouraud agar was adjusted to pH 4.0 and supplemented with 10% (v/v) cell-free supernatant.
MRS – de Man, Rogosa and Sharpe broth containing 3% glucose; sMRS – MRS broth supplemented with 0.5 g·l^{-1} phenylalanine, 0.1 g·l^{-1} tyrosine and 0.2 g·l^{-1} α-ketoglutarate; *E. coli* – MRS broth supplemented with 200 mmol·l^{-1} glycerol and inactivated cells of *E. coli* K12.

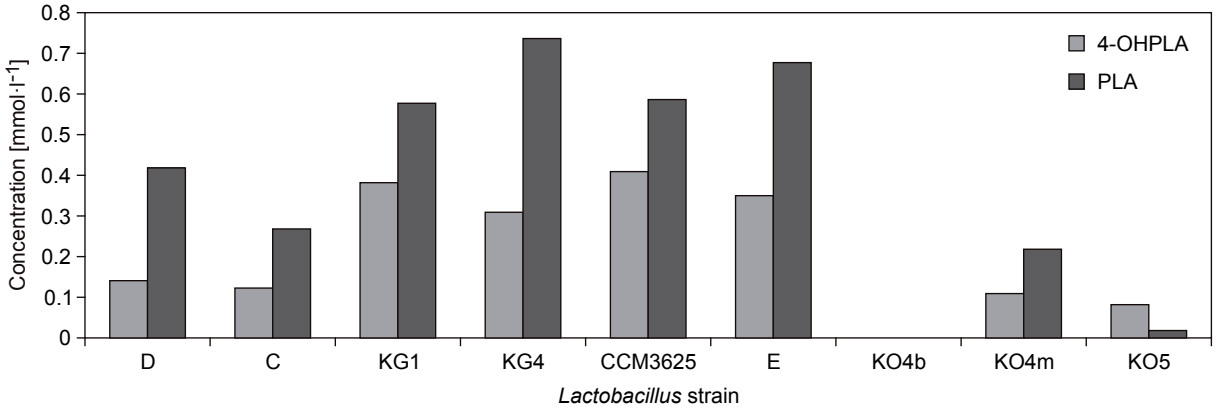

Fig. 2. Production of 4-hydroxyphenyllactic acid and 3-phenyllactic acid by lactotobacilli.

Lactobacilli were cultured in de Man, Rogosa and Sharpe broth supplemented with 0.5 g·l^{-1} phenylalanine, 0.1 g·l^{-1} tyrosine and 0.2 g·l^{-1} α-ketoglutarate for 72 h at 37 °C.
4-OHPLA – 4-hydroxyphenyllactic acid, PLA – 3-phenyllactic acid, D – *Lb. mucosae* D, C – *Lb. murinus* C, KG1 – *Lb. plantarum* KG1, KG4 – *Lb. plantarum* KG4, CCM3625 – *Lb. reuteri* CCM3625, E – *Lb. reuteri* E, KO4b – *Lb. reuteri* KO4b (no PLA or 4-OHPLA detected), KO4m – *Lb. reuteri* KO4m, KO5 – *Lb. reuteri* KO5.

The highest amount of PLA was produced by *Lb. plantarum* KG4 (0.7400 ± 0.0217 mmol·l^{-1}) and *Lb. reuteri* E (0.6800 ± 0.0224 mmol·l^{-1}). The highest production of 4-OHPLA was observed in *Lb. reuteri* CCM3625 (0.4000 ± 0.0126 mmol·l^{-1}). The amounts of PLA produced with our strains in MRS supplemented with Phe, Tyr and α-KG were not as high as that reported by Valerio et al. [34] with phenylpyruvic acid supplementation of defined growth media (0.5–2.4 mmol·l^{-1}). However, supplementation with amino acids increased the production of PLA and 4-OHPLA to a higher level than by *Lb. plantarum* CRL778 (0.26 mmol·l^{-1} and 0.28 mmol·l^{-1}, respectively), as reported by Dallagnol et al. [30]. Cortés-Zavaleta et al. [31] observed that higher production of PLA was not inevitably associated with high antifungal activity. *Lb. plantarum* KG4, as the best producer of PLA among the tested strains, and *Lb. reuteri* KO4b, which did not produce any PLA, showed a similar antifungal activity in our study. The antifungal activity of tested strains is predominantly related to its metabolites, especially to organic acids, according to results with CFS. Thus, PLA is not necessarily the key compound produced by lactobacilli that is responsible for inhibition of fungi

Ability of lactobacilli to bind AFB_1 in vitro

We tested our new isolates and one collection strain for ability to bind AFB_1 in vitro according to the method published by El-Nezami [16].

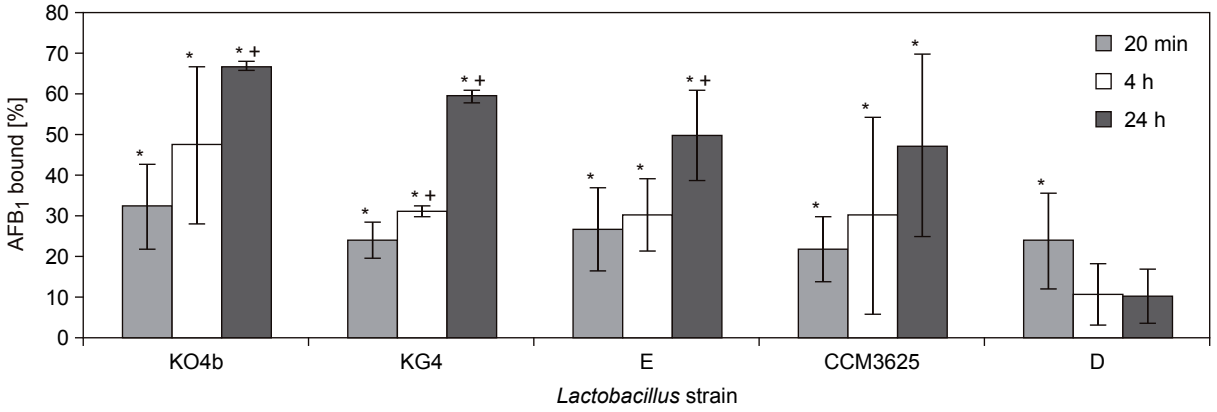

Fig. 3. Portion of AFB_1 bound to lactobacilli after different incubation time.

(*) – significant difference between positive control and sample, (+) – significant difference between two time intervals within the strain ($p < 0.05$).
KO4b – *Lb. reuteri* KO4b, KG4 – *Lb. plantarum* KG4, E – *Lb. reuteri* E, CCM3625 – *Lb. reuteri* CCM3625, D – *Lb. mucosae* D.

In this work, five isolates were tested regarding their ability to bind AFB_1 in vitro. Three strains (*Lb. reuteri* E, *Lb. reuteri* KO4b and *Lb. plantarum* KG4) were selected according to their antifungal activity. Strain *L. mucosae* D was selected for its ability to bind to intestinal mucosa (our unpublished results). *Lb. reuteri* CCM 3625 was used as a reference strain. Binding activity was evaluated at three time intervals. Results given in Fig. 3 showed that all the analysed lactobacilli were able to bind AFB_1. The extent of binding ability was directly proportional to the length of incubation with AFB_1 for all strains with the exception of *Lb. mucosae* D strain, which showed a decreased activity with time. The best results concerning AFB_1 reduction and reproducibility of the reduction process were obtained with *Lb. reuteri* KO4b strain (66.7 % ± 1.0 %) followed by *Lb. plantarum* KG4 (59.4 % ± 1.6 %) after an incubation of 24 h. Previous investigations on aflatoxin binding were carried out mainly with *Lb. rhamnosus, Lb. casei, Lb. acidophilus, Lb. amylovorus,* and *Lb. brevis* [19]. Hernandez-Mendoza et al. [18] reported 79.0 % of bound AFB_1 by *Lb. reuteri* NRRL14171 after an incubation of 4 h. Fazeli et al. [35] reported 56.0 % of bound AFB_1, from a solution, by *Lb. plantarum*. These data are in accordance with our results, as *Lb. plantarum* KG4 bound 59.4 % ± 1.6 % of AFB_1 in our study. In previous studies, lactobacilli eliminated fungal and bacterial contamination and mycotoxin production by toxic fungal strains [23, 36, 37]. It was proven that addition of lactobacilli to feed decreased the uptake of toxins from food and had protective effect on animals after toxin consumption [38]. Also, lactic acid produced by LAB transformed aflatoxin B_1 at a temperature of 80 °C to less toxic AFB_{2a} or AFB_2 [39]. Here we firstly reported an ability to bind AFB_1 and antifungal activity by strains with lamb and goatling origin. The strains could be a possible supplementation of feed as a biological conservation agent. Our strains, isolated from stomach mucus, could be adapted to ruminal environment. If confirmed to have probiotic properties, they could serve also as probiotic additives.

CONCLUSIONS

In this study a dual activity, i.e. antifungal activity and ability to bind aflatoxin B_1, of the strains isolated from lamb and goatling was investigated. The tested lactobacilli exhibited ability to inhibit growth of seven fungal species. The degree of inhibition was found to be strain-dependent. Selected strains were tested also for their ability to bind AFB_1 and two strains, namely, *Lb. reuteri* KO4b and *Lb. plantarum* KG4 exhibited reasonable ability to bind AFB_1 in vitro. Hence, these two strains are potential candidates for application in biological conservation. CFS from *Lb. reuteri* E, *Lb. reuteri* KO5, *Lb. reuteri* KO4b and *Lb. plantarum* KG4 with the strong antifungal properties could be appropriate for the treatment of different food or raw materials in biological control of fungal spoilage. The impact of presence of our strains in raw materials on fungal growth and production of mycotoxins, as well as on in vivo uptake of mycotoxins in animal gut influenced by our lactobacilli strains, would be evaluated in the future, to evaluate these strains regarding their suitability for biological conservation.

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